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(54) STEVIOL GLYCOSIDE TRANSPORT

STEVIOL GLYCOSIDE TRANSPORT

TRANSPORT DE GLYCOSIDES DE STEVIOL

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WO-A1-2014/122328 WO-A1-2015/011209
WO-A2-2014/191581

- **DATABASE UniProt [Online] 16 August 2004 (2004-08-16), "SubName: Full=YAL0E25201p", XP002763706, Database accession no. Q6C4M7**

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Description

Field of the invention

[0001] The present invention relates to a recombinant host capable of producing a steviol glycoside. The invention also relates to a process for the preparation of a steviol glycoside using such a recombinant host. The invention also relates to a fermentation broth comprising a steviol glycoside, a steviol glycoside and to a composition comprising two or more steviol glycosides. The invention further relates to a foodstuff, feed or beverage which comprises a steviol glycoside or a composition comprising two or more steviol glycosides.

Background

[0002] The leaves of the perennial herb, *Stevia rebaudiana* Bert., accumulate quantities of intensely sweet compounds known as steviol glycosides. Whilst the biological function of these compounds is unclear, they have commercial significance as alternative high potency sweeteners.

[0003] These sweet steviol glycosides have functional and sensory properties that appear to be superior to those of many high potency sweeteners. In addition, studies suggest that stevioside can reduce blood glucose levels in Type II diabetics and can reduce blood pressure in mildly hypertensive patients.

[0004] Steviol glycosides accumulate in Stevia leaves where they may comprise from 10 to 20% of the leaf dry weight. Stevioside and rebaudioside A are both heat and pH stable and suitable for use in carbonated beverages and many other foods. Stevioside is between 110 and 270 times sweeter than sucrose, rebaudioside A between 150 and 320 times sweeter than sucrose. In addition, rebaudioside D is also a high-potency diterpene glycoside sweetener which accumulates in Stevia leaves. It may be about 200 times sweeter than sucrose. Rebaudioside M is a further high-potency diterpene glycoside sweetener. It is present in trace amounts in certain stevia variety leaves, but has been suggested to have a superior taste profile.

[0005] Steviol glycosides have traditionally been extracted from the *Stevia* plant. In *Stevia,* (-)-kaurenoic acid, an intermediate in gibberellic acid (GA) biosynthesis, is converted into the tetracyclic diterpene steviol, which then proceeds through a multi-step glycosylation pathway to form the various steviol glycosides. However, yields may be variable and affected by agriculture and environmental conditions. Also, Stevia cultivation requires substantial land area, a long time prior to harvest, intensive labour and additional costs for the extraction and purification of the glycosides.

[0006] More recently, interest has grown in producing steviol glycosides using fermentative processes. WO2013/110673 and WO2015/007748 describe microorganisms that may be used to produce at least the steviol glycosides rebaudioside A, rebaudioside D and rebaudioside M.

[0007] WO2014122328 relates to production of steviol glycoside in recombinant hosts. Recombinant microorganisms are disclosed that produce steviol glycosides and have altered expression of one or more endogenous transporter or transcription factor genes, or that overexpress one or more heterologous transporters, leading to increased excretion of steviol glycosides of interest.

[0008] WO2014191581 relates to a recombinant microorganism comprising one or more nucleotide sequence(s) encoding: a polypeptide having ent-copalyl pyrophosphate synthase activity; a polypeptide having ent-Kaurene synthase activity; a polypeptide having ent-Kaurene oxidase activity; and a polypeptide having kaurenoic acid 13-hydroxylase activity, whereby expression of the nucleotide sequence(s) confer(s) on the microorganism the ability to produce at least steviol, and wherein said recombinant microorganism has been modified in its genome such that it results in a deficiency in the production of one or more of: (i) a phosphatase capable of acting on geranylgeranylpyrophosphate (GGPP) resulting in the formation of geranylgeraniol (GOH); (ii) a phosphatase capable of acting on farnesylpyrophosphate (FPP) resulting in the formation of farnesol and nerolidol;(iii) an exo-1,3-βglucanase;(iv)a glycogen synthase (or a polypeptide that influences glycogen accumulation); (v) a transcriptional repressor of hypoxic genes (ROX1) (vi) an NADPH oxidase; or (vii) a monocarboxylate transporter (JEN1) (viii) a polypeptide having activity as encoded for by the open reading frame, YJL064w; or (ix) a polypeptide. The recombinant microorganism may also be capable of expressing one or more UDP-glucosyltransferases such that the microorganism is capable of producing one or more steviol glycosides.

[0009] WO2015011209 relates to a method for the production of a diterpene or a glycosylated diterpene, which method comprises: a. fermenting a recombinant microorganism of the genus Yarrowia in a suitable fermentation medium at a temperature of about 29°C or higher, wherein the microorganism comprises one or more nucleotide sequence(s) encoding: a polypeptide having ent-copalyl pyrophosphate synthase activity; a polypeptide having ent-Kaurene synthase activity; a polypeptide having ent-Kaurene oxidase activity; and a polypeptide having kaurenoic acid 13-hydroxylase activity and whereby expression of the nucleotide sequence(s) confer(s) on the microorganism the ability to produce at least steviol; and b. recovering the diterpene or glycosylated diterpene.

[0010] Further improvement of such microoganisms is desirable in order that higher amounts of steviol glycosides may be produced and/or additional or new steviol glycosides and/or higher amounts of specific steviol glycosides and/or mixtures of steviol glycosides having desired ratios of different steviol glycosides.

Summary

[0011] The present invention is based on the identification of a protein which is capable of mediating steviol glycoside transport.

[0012] Accordingly, the protein may be overexpressed in a recombinant host (such as a microbial cell) in order to increase steviol glycoside transport out of the host. Alternatively, a host (such as a microbial cell) may be modified so as to express less of the protein than a corresponding non-modified version of the host. In this case, more steviol glycoside may be retained within the host which is then glycosylated to a steviol glycoside comprising a higher number of sugar moieties.

[0013] Thus, the present disclosure relates to a recombinant yeast host, which produces steviol glycoside outside the host to a greater degree than a corresponding host not overexpressing the protein. This may facilitate easier recovery of steviol glycosides. The disclosure also relates to a recombinant yeast host capable of producing a steviol glycoside which overexpresses a heterologous polypeptide which mediates steviol glycoside transport.

[0014] Accordingly, the invention relates to a recombinant yeast host capable of producing a steviol glycoside which overexpresses a polypeptide which mediates steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least about 70% sequence identity thereto.

[0015] The invention also relates to a recombinant Yarrowia host capable of producing a steviol glycoside which has been modified, preferably in its genome, to result in a deficiency in the production of a polypeptide which mediates steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least about 70% sequence identity thereto.

[0016] The present disclosure also relates to a recombinant host which comprises steviol glycosides (inside and/or outside the host) having a higher or lower average glycosylation number than a corresponding host not modified according to the invention.

[0017] The invention also relates to a process for the preparation of a steviol glycoside which comprises fermenting a recombinant host according to the invention in a suitable fermentation medium and, optionally, recovering the steviol glycoside.

[0018] The disclosure also relates to:

a fermentation broth comprising a steviol glycoside obtainable by a process of the invention;
a steviol glycoside obtained by a process or a fermentation broth a composition comprising two or more steviol glycosides or obtainable by a process of the invention;
a foodstuff, feed or beverage which comprises a steviol glycoside or a composition.

Brief description of the drawings

[0019]

Figure 1 sets out a schematic representation of the plasmid MB6969, encoding tHMG, UGT2_1a, HPH.
Figure 2 sets out a schematic representation of the plasmid MB6856, encoding tHMG.
Figure 3 sets out a schematic representation of the plasmid MB6857, encoding tHMG.
Figure 4 sets out a schematic representation of the plasmid MB6948, encoding GGS.
Figure 5 sets out a schematic representation of the plasmid MB6958, encoding GGS.
Figure 6 sets out a schematic representation of the plasmid MB7015, encoding UGT1, UGT3, UGT4, NAT.
Figure 7 sets out a schematic representation of the plasmid MB6986, encoding tHMG, URA3, GGS.
Figure 8 sets out a schematic representation of the plasmid MB7059, encoding tCPS_SR, tKS_SR, KAH_4, KO_Gib, CPR_3, LEU2.
Figure 9 sets out a schematic representation of the plasmid MB7100, encoding tCPS_SR, tKS_SR, KAH_4, KO_Gib, CPR_3, URA3.
Figure 10 sets out a schematic representation of the plasmid MB6988, encoding tHMG, URA2, GGS.
Figure 11 sets out a schematic representation of the plasmid MB7044, encoding tCPS_SR, tKS_SR, KAH_4, KO_Gib, CPR_3, LEU2.
Figure 12 sets out a schematic representation of the plasmid MB7094, encoding tCPS_SR, tKS_SR, KAH_4, KO_Gib, CPR_3, URA2.
Figure 13 sets out a schematic representation of the plasmid MB6128, encoding CRE, neoR.
Figure 14 sets out a schematic representation of the construct containing KAH and HPH.
Figure 15 sets out a schematic representation of the construct containing tCPS_SR.

Figure 16 sets out a schematic representation of the plasmid MB6986, encoding tHMG, URA3, GGS.

Figure 17 sets out a schematic representation of the plasmid pRS417 Con5-3.

Figure 18 sets out a schematic representation of the assembly of the HygB marker with the transporter internal fragments in plasmid pRS417 5-3.

Figure 19 sets out a schematic representation of the PCR amplification of the transporter disruption constructs off plasmid pRS417 5-3 containing the HYG marker and transporter internal fragments.

Figure 20 sets out a schematic representation of the recombination event at the genome resulting in a disruption of the transporter gene and integration of the HygB marker.

Figure 21 sets out a schematic diagram of the potential pathways leading to biosynthesis of steviol glycosides. The compound shown with an asterisk is 13-[(β-D-Glucopyranosyl)oxy)kaur-16-en-18-oic acid 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester.

Description of the sequence listing

[0020] A description of the sequences is set out in Table 14. Sequences described herein may be defined with reference to the sequence listing or with reference to the database accession numbers also set out in Table 14.

Detailed description

[0021] Throughout the present specification and the accompanying claims, the words "comprise", "include" and "having" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

[0022] The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

[0023] The invention is embodied in the attached claims.

[0024] The disclosure relates to the identification of a polypeptide which is capable of mediating steviol glycoside transport. Such a polypeptide may directly mediate steviol glycoside transport, i.e. may be a transporter protein, or may indirectly mediate steviol glycoside transport. Such a polypeptide may be capable of mediating transport of one or more steviol glycoside.

[0025] The disclosure relates to a recombinant yeast host either overexpressing or having reduced expression of such a polypeptide. The terms recombinant host or recombinant cell may, depending on the context, be used interchangeably.

[0026] Such a polypeptide as described herein may be overexpressed in a recombinant host, such as a recombinant host cell, capable of producing one or more steviol glycosides. Such a cell may be capable of producing more of one or more steviol glycosides external to the cell than a corresponding cell which does not overexpress the polypeptide. That is to say, a recombinant yeast cell according to the invention may have increased or decreased steviol glycoside transport in a comparison with a corresponding non-recombinant cell .

[0027] Accordingly, the invention provides a recombinant yeast host capable of producing a steviol glycoside which overexpresses a polypeptide, the polypeptide being one which is capable of mediating steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least about 70% sequence identity thereto.

[0028] The expression of such a polypeptide may also be modified in a host, such as a recombinant host cell, such that it is reduced compared to a corresponding cell which has not been similarly modified. In this way, the amount of one or more steviol glycosides outside the cell may be reduced in comparison with a corresponding cell which has not been similarly modified. This may allow for increased glycosylation of one or more steviol glycosides within the cell compared with a corresponding cell which has not been similarly modified. Such a host may thus comprise steviol glycosides having a higher average glycosylation number compared with a corresponding cell which has not been similarly modified.

[0029] Accordingly, the invention provides a recombinant Yarrowia host capable of producing a steviol glycoside which has been modified, preferably in its genome, to result in a deficiency in the production of a polypeptide, the polypeptide being one which is capable of mediating steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least about 70% sequence identity thereto.

[0030] A host cell of the invention is a recombinant yeast host cell. "Recombinant" in this sense means that the host cell is a non-naturally occurring host cell, for example modified by introduction of one or more nucleic acids using recombinant techniques. A nucleic acid used to modify a host cell to arrive at a recombinant host cell of the invention may be a naturally-occurring nucleic acid or a non-naturally occurring nucleic acid.

[0031] Thus, when used in reference to a host of the invention, "recombinant" indicates that a cell has been modified by the introduction of one or more heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. The term "heterologous" as used herein refers to nucleic acid or amino acid sequences not naturally occurring in a host cell. In other words, the nucleic acid or amino acid sequence is not identical to that naturally found in the host cell.

[0032] The disclosure relates to a recombinant yeast host capable of producing a steviol glycoside which overexpresses a heterologous polypeptide which mediates steviol glycoside transport. Such a heterologous polypeptide may be obtained from or derived from a genus or species other than that of the host. Accordingly, the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from a different genus or species of yeast.

[0033] For example, if the host cell is a *Saccharomyces* (e.g., *S. cerevisiae, S. bayanus, S. pastorianus, S. carlsbergensis),* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from a *Candida* (e.g., *C. krusei, C. revkaufi, C. pulcherrima, C.tropicalis, C. utilis),* an *Issatchenkia* (eg. *I. orientalis)* or a *Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica)).*

[0034] For example, if the host cell is a *Candida* (e.g., *C. krusei, C. revkaufi, C. pulcherrima, C. tropicalis, C. utilis),* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from a *Saccharomyces* (e.g., *S. cerevisiae, S. bayanus, S. pastorianus, S. carlsbergensis),* an *Issatchenkia* (eg. *I. orientalis)* or a *Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica)).*

[0035] For example, if the host cell is an *Issatchenkia* (eg. *I. orientalis),* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from a *Saccharomyces* (e.g., *S. cerevisiae, S. bayanus, S. pastorianus, S. carlsbergensis), a Candida* (e.g., *C. krusei, C. revkaufi, C. pulcherrima, C. tropicalis, C. utilis)* or a *Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica)).*

[0036] For example, if the host cell is a *Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica)),* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from a *Saccharomyces* (e.g., *S. cerevisiae, S. bayanus, S. pastorianus, S. carlsbergensis). a Candida* (e.g., *C. krusei, C. revkaufi, C. pulcherrima, C. tropicalis, C. utilis)* or an *Issatchenkia* (eg. *I. orientalis).*

[0037] If the host cell is *Saccharomyces cerevisiae,* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from *Yarrowia lipolytica* (formerly classified as *Candida lipolytica)), Candida krusei* or *Issatchenkia orientalis.*

[0038] If the host cell is *Yarrowia lipolytica,* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from *Saccharomyces cerevisiae, Yarrowia lipolytica* (formerly classified as *Candida lipolytica))* or *Candida krusei* or *Issatchenkia orientalis.*

[0039] If the host cell is *Candida krusei* or *Issatchenkia orientalis,* the heterologous polypeptide which mediates steviol glycoside transport may be obtained from or derived from *Saccharomyces cerevisiae* or *Yarrowia lipolytica.*

[0040] The term "derived from" also includes the terms "originated from," "obtained from," "obtainable from," "isolated from," and "created from," and generally indicates that one specified material find its origin in another specified material or has features that can be described with reference to the another specified material. As used herein, a substance (e.g., a nucleic acid molecule or polypeptide) "derived from" a microorganism may indicate that the substance is native to that microorganism or is a substance native to that microorganism, but may also indicate a substance that has been altered from a native version.

[0041] Thus, for example, a recombinant cell may express a polypeptide as defined herein not found within the native (non-recombinant) form of the cell. Alternatively, a recombinant cell may be modified so as to express a native gene encoding a polypeptide as defined herein to a greater degree than takes place within the native "non-recombinant" form of the cell.

[0042] Alternatively, a recombinant cell may be modified so as to express a native gene encoding a polypeptide as defined herein to a lesser degree than takes place within the native "non-recombinant" form of the cell.

[0043] In a cell of the invention, a polypeptide as defined herein may be overexpressed. Herein, "overexpressed", "overexpression" or the like implies that the recombinant host cell expresses more of the polypeptide than a corresponding cell which does not overexpress the polypeptide or, alternatively, that the polypeptide is expressed in a cell which would not typically express that protein. Alternatively, overexpression may be achieved by expressing a variant polypeptide having a higher specific activity.

[0044] A recombinant yeast cell of the invention cell may be modified, preferably in its genome, to result in a deficiency in the production of a polypeptide as defined herein.

[0045] Such a cell may be from a parent host cell and be modified, preferably in its genome, if compared to the parent host cell to obtain a different genotype and/or a different phenotype if compared to the parent host cell from which it is derived.

[0046] Such a cell which has been modified, preferably in its genome, to result in a deficiency in the production of a polypeptide as defined herein, is a mutant host cell which has been modified, preferably in its genome, to result in a phenotypic feature wherein the cell: a) produces less of the product or produces substantially no product and/or b) produces a product having a decreased activity or decreased specific activity or a product having no activity or no specific activity and combinations of one or more of these possibilities as compared to the parent microbial host cell that has not been modified, when analyzed under the same conditions.

[0047] The term "recombinant" is synonymous with "genetically modified".

[0048] Such a recombinant host may be a full or partial knock-out of a nucleic acid sequence encoding a polypeptide as described herein.

[0049] The disclosure thus concerns recombinant yeast hosts overexpressing or deficient in a polypeptide identified as having steviol glycoside transport mediating activity: typically, the host is one which may be used for the production of steviol glycosides. The ability of a given recombinant host to produce a steviol glycoside may be a property of the host in non-recombinant form or may be a result of the introduction of one or more recombinant nucleic acid sequences (i.e. encoding enzymes leading to the production of a steviol glycoside).

[0050] For the purpose of this invention, a polypeptide having steviol glycoside transport mediating activity (i.e. a polypeptide which mediates steviol glycoside transport) is one which has an effect on transport of one or more steviol glycosides across a cell membrane. The effect may be direct, i.e. the polypeptide may be a transporter protein or comprise a functional transporter region. Alternatively, the effect may be indirect, i.e. the polypeptide is not a transporter protein, but its activity nevertheless has an effect on steviol glycoside transport.

[0051] Typically, the effect will be such that increasing the level of expression of the polypeptide increases the amount of transport of one or more steviol glycosides across the membrane of a cell (in comparison with a corresponding cell having a lower level of expression of the polypeptide). Conversely, decreasing the level of expression of the polypeptide may decrease the amount of transport of one or more steviol glycosides across the membrane of a cell (in comparison with a corresponding cell having a higher level of expression of the polypeptide).

[0052] Typically, a recombinant yeast host of the invention is capable of producing a steviol glycoside. For example, a recombinant host of the invention may be capable of producing one or more of, for example but not limited to, steviol-13-monoside, steviol-19-monoside, 13-[(β-D-Glucopyranosyl)oxy)kaur-16-en-18-oic acid 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester, rubusoside, stevioside, steviol-19-diside, steviolbioside, rebA, rebB, rebC, rebD, rebE or rebM. A recombinant host of the invention may be capable of producing one or more of the steviol glycosides set out in Ceunen and Geuns, Journal of Natural Products 76(6), 1201-1228, 2013.

[0053] Thus, a yeast cell of the invention may be one in which the amount of total amount of steviol glycosides outside the cell as compared with inside the cell is greater or less than compared with a corresponding cell which either does not overexpress or does not have a reduced level of expression of a cell of the invention.

[0054] Alternatively, a yeast cell of the invention may have the same total amount of steviol glycosides outside the cell as compared with inside the cell compared with a corresponding cell which either does not overexpress or does not have a reduced level of expression of a cell of the invention, but may have an altered distribution of steviol glycosides inside and outside the cell.

[0055] Thus, a recombinant yeast host of the invention is capable of producing a steviol glycoside. For example, a recombinant host of the invention may be capable of producing one or more of, for example, steviol-13-monoside, steviol-19-monoside, 13-[(β-D-Glucopyranosyl)oxy)kaur-16-en-18-oic acid 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester, rubusoside, stevioside, steviol-19-diside, steviolbioside, rebA, rebB, rebC, rebD, rebE or rebM.

[0056] Thus, a recombinant yeast host of the invention may be one in which at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50% of the rebA produced by the cell is outside the cell.

[0057] Thus, a recombinant yeast host of the invention may be one in which at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50% of the rebD produced by the cell is outside the cell.

[0058] Thus, a recombinant yeast host of the invention may be one in which at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50% of the rebM produced by the cell is outside the cell.

[0059] A recombinant yeast cell of the invention may be one in which no more than about 50%, no more than about 40%, no more than about 30%, no more than about 20%, no more than about 10% of the rebA produced by the cell is outside the cell.

[0060] A recombinant yeast cell of the invention may be one in which no more than about 50%, no more than about 40%, no more than about 30%, no more than about 20%, no more than about 10% of the rebD produced by the cell is outside the cell.

[0061] A recombinant yeast cell of the invention may be one in which no more than about 50%, no more than about 40%, no more than about 30%, no more than about 20%, no more than about 10% of the rebM produced by the cell is outside the cell.

[0062] A recombinant yeast cell of the invention may be one where the average glycosylation number of the steviol glycosides is at least 3, at least 4, at least 5, at least 6 or more. The average glycosylation number may be increased or decreased in comparison with a corresponding cell not modified according to the invention. For example, average glycosylation may decrease when a polypeptide as described herein is overexpressed. For example, average glycosylation may increase (in particular in a cell itself) when expression of a polypeptide of the invention is reduced.

[0063] The average glycosylation may refer to that in the supernatant of a recombinant yeast cell of the invention or to the average glycosylation in the broth (pellet + supernatant).

[0064] The invention thus provides a recombinant yeast cell capable of producing a steviol glycoside either overexpressing or deficient in the expression of a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least about 70% sequence identity thereto. Such an amino acid sequence has an effect of steviol glycoside transport, i.e. is a mediator of steviol glycoside transport.

[0065] The polypeptide may also be defined as one comprising the following amino acid sequence (or an amino acid sequence having at least about 45% sequence identity thereto):

MGKTEVTQESLECGSVTSSLGKKPFSIITLFTGRRIPPVPTEKPDSAEERAGILSKLTWQWLSPLL
KTGYLRNIEREDLYKVRERNSAAVIQQRLESNLEKQYAKYHAKLLKKGLSEQEAHLKLQDSAKPL
VLALNQTFFWKFWLAGLFALVKDLCGIASAMVSRVLIEYIQDRYLYRGTDREPKVGRGVGPSIGL
FLLAVGVTFFFNHMFYNVKMVGAQARAALVAVIYSKSTRLSAKGRAQYTTGKITNLAAIDAHRVDL
SCESFHYITIFLPVVGCAIAVLVVNLKVAALVGIATMIVLIFVVAGITIFSMKLRAIIVKLTDKRVTYIRE
ALQSIRIIKYYGWEVPYCDKIKKVRLDETRNYAKMGSIRGTAIGMFQALPILAGALSFITYAALGHG
TDPARMFSSLTLFNLLLPALAVLPQALQAAGDARVALRRIQRFLGAEESTPTTVFDATLESTDDAV
IVEDASFIWPEVVDDKSDKEKAKDAKKEEKDKKKAEKKAKKAAKKAAKEIAVVVEEEVEHEKTEG
SSESEKGTLKSTFKGFNNLSFKIKRGEFVVVTGPIGSGKSSLLAAITGSMVLTGGSVRVSSTEWIG
CLEPWIQNATVRDNIVFGRKFDSEWYRTVVTACQLSQDLKIMTHGDNTMIGERGITVSGGQKARI
NLARAIYGNPEILIMDDVLSAVDARVGAGIVDDCLRGLAKNSTRILATHQLSVLPKADHVIFMDAEG
QFHIGTYQELEADNEQFKALLAAGSMSKEEVVAVDETEVVIEGDLEDDCDNKEEYEDAAETISILA
DATQELQKVTTTVSAFEENDNMMEEEERMRDAVGLHVYWQYFRQANPSRVKVMMFIGMIFISMI
VIAFLFVFTSVWLSFWTGDRFHASRNFYTGIYIMLGILLLLAVAGYMIVNEINSAMAARNLHNHALD
SVFAARTSFFDTTPQGRIINRFTRDTDSLDNELAMRLTMLFFGVSAFFSNFLLTCVYVPYVTLVLV
PVGFVFYVSLGYYRKSAREVKRIDSIERSHMMSVFNESISGMPVIIMYKAQHRLMNKLQATLDDM
DSAYFLTAANQRWLSLRLDGLGSLVVLVATILVAVGVFDLTPSNMGLIISAASFIPEVMSMVAQAV
AELENCMNATERILYYKDNIPAEAAREVDGTELDQRPNWPEQGAISFNNVSMKYRDGLPYVLKS
LSVDFQGGHKVGICGRTGAGKSTILQTLYRIVELAEGSITIDGVDISTIGLHQLRSQLSIIPQEPVLFL

GTIRSNLDPLEQYSDAELWGSLRRSGLLDEGETEGKFHLDQKVEADGSNFSLGERQLLTLARAL
LRNTKILVLDEATSNVDYKTDKLVQETISREFGHCTILCIAHRLRTIAKYDRILVLESGEINQYDTPW
NLYNDKEGIFRGMCDTSGLNEVDFNK (SEQ ID NO: 29).

[0066] A polypeptide, typically having steviol glycoside transport mediating activity, may comprise an amino acid sequence having at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to SEQ ID NO: 29.

[0067] A polypeptide, typically having steviol glycoside transport mediating activity, encoded by a recombinant nucleic acid present in a recombinant yeast host of the invention may comprise an amino acid sequence which is a fragment of an amino acid sequence described herein, for example a truncated version of such an amino acid sequence.

[0068] That is to say, the disclosure also relates to a recombinant yeast host overexpressing a biologically active fragment of a polypeptide having steviol glycoside transport mediating activity as described herein.

[0069] Biologically active fragments of a polypeptide include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of SEQ ID NO: 29 which include fewer amino acids than the full-length polypeptide as given in SEQ ID NO: 29, but which exhibit at least one biological activity of the corresponding full-length polypeptide.

[0070] Typically, biologically active fragments comprise a domain or motif with at least one activity of the polypeptide. A biologically active fragment of a polypeptide can be a polypeptide which is, for example, about 10, about 25, about 50, about 100 or more amino acids in length or at least about 100 amino acids, at least 150, 200, 250, 300, 350, 400, 600, 1000 amino acids in length, or of a length up to the total number of amino acids of the polypeptide. Moreover, other biologically active portions, in which other regions of the polypeptide are deleted, can be prepared by recombinant techniques and evaluated for one or more of the biological activities of the native form of a polypeptide. The disclosure also features nucleic acid fragments which encode the above biologically active fragments of the polypeptide. A recombinant yeast host may overexpress or be deficient in such a polypeptide.

[0071] A recombinant yeast host may comprise recombinant nucleic acid sequences encoding more than one such polypeptide, for example two, three, four or more such polypeptides. The polypeptides thus encoded may be the same or different.

[0072] A recombinant yeast cell may be modified so as to reduce the expression level of more than one such polypeptide, for example two, three, four or more such polypeptides.

[0073] An overexpressed polypeptide encoded by a recombinant nucleic acid present in a recombinant host may be one which is obtainable from or derived from or found in an organism of the genus *Yarrowia,* for example one which is obtainable from or derived from or found in a *Yarrowia lipolytica.*

[0074] As used herein, the term "polypeptide" refers to a molecule comprising amino acid residues linked by peptide bonds and containing more than five amino acid residues. The amino acids are identified by either the single-letter or three-letter designations. The term "protein" as used herein is synonymous with the term "polypeptide" and may also refer to two or more polypeptides. Thus, the terms "protein", "peptide" and "polypeptide" can be used interchangeably. Polypeptides may optionally be modified (e.g., glycosylated, phosphorylated, acylated, farnesylated, prenylated, sulfonated, and the like) to add functionality. Polypeptides exhibiting activity may be referred to as enzymes. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given polypeptide may be produced.

[0075] A polypeptide encoded by a recombinant nucleic acid for use in a recombinant host of the invention may comprise a signal peptide and/or a propeptide sequence. In the event that a polypeptide comprises a signal peptide and/or a propeptide, sequence identity may be calculated over the mature polypeptide sequence.

[0076] A recombinant nucleic acid sequence for use in a recombinant yeast host of the invention may be provided in the form of a nucleic acid construct. The term "nucleic acid construct" refers to a nucleic acid molecule, either single-or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains all the control sequences required for expression of a coding sequence, wherein said control sequences are operably linked to said coding sequence.

[0077] A recombinant nucleic acid sequence for use in a recombinant yeast host of the invention may be provided in the form of an expression vector, wherein the polynucleotide sequence is operably linked to at least one control sequence for the expression of the polynucleotide sequence in a recombinant host cell.

[0078] The term "operably linked" as used herein refers to two or more nucleic acid sequence elements that are physically linked and are in a functional relationship with each other. For instance, a promoter is operably linked to a coding sequence if the promoter is able to initiate or regulate the transcription or expression of a coding sequence, in which case the coding sequence should be understood as being "under the control of" the promoter. Generally, when two nucleic acid sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They usually will be essentially contiguous, although this may not be required.

[0079] An expression vector comprises a polynucleotide coding for a polypeptide as described herein, operably linked to the appropriate control sequences (such as a promoter, and transcriptional and translational stop signals) for expression and/or translation *in vitro,* or in the host cell of the polynucleotide.

[0080] The expression vector may be any vector (e.g., a plasmid or virus), which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, i.e., a vector, which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome.

[0081] Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The integrative cloning vector may integrate at random or at a predetermined target locus in the chromosomes of the host cell. A vector may comprise one or more selectable markers, which permit easy selection of transformed cells.

[0082] A recombinant host capable of producing a steviol glycoside which has been modified, preferably in its genome, to result in a deficiency in the production of a polypeptide described herein may be generated according to methods well known to those skilled in the art. A sequence encoding a polypeptide as described herein may be modified such that less or no expression of the polypeptide takes place. A sequence encoding a polypeptide as described herein may be partially or entirely deleted, for example.

[0083] A recombinant yeast host may comprise any polypeptide as described herein. A recombinant host may overexpress or be deficient in any polypeptide described herein. Typically, a recombinant host of the invention is capable of producing a steviol glycoside. For example, a recombinant host of the invention may be capable of producing one or more of, for example, steviol-13-monoside, steviol-19-monoside, 13-[(β-D-Glucopyranosyl)oxy)kaur-16-en-18-oic acid 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester, rubusoside, stevioside, steviol-19-diside, steviolbioside, rebA, rebE, rebD or rebM.

[0084] A recombinant yeast host of the invention may comprise one or more recombinant nucleic acid sequences encoding one or more polypeptides having UDP-glycosyltransferase (UGT) activity.

[0085] For the purposes of this invention, a polypeptide having UGT activity is one which has glycosyltransferase activity (EC 2.4), i.e. that can act as a catalyst for the transfer of a monosaccharide unit from an activated nucleotide sugar (also known as the "glycosyl donor") to a glycosyl acceptor molecule, usually an alcohol. The glycosyl donor for a UGT is typically the nucleotide sugar uridine diphosphate glucose (uracil-diphosphate glucose, UDP-glucose).

[0086] Such additional UGTs may be selected so as to produce a desired steviol glycoside. Schematic diagrams of steviol glycoside formation are set out in Humphrey et al., Plant Molecular Biology (2006) 61: 47-62 and Mohamed et al., J. Plant Physiology 168 (2011) 1136-1141. In addition, Figure 21 sets out a schematic diagram of steviol glycoside formation.

[0087] A recombinant yeast host of the invention may thus comprise one or more recombinant nucleic acid sequences encoding one or more of:

(i) a polypeptide having UGT74G1 activity;
(ii) a polypeptide having UGT2 activity;
(iii) a polypeptide having UGT85C2 activity; and
(iv) a polypeptide having UGT76G1 activity.

[0088] A recombinant yeast suitable for use in the invention may comprise a nucleotide sequence encoding a polypeptide capable of catalyzing the addition of a C-13-glucose to steviol. That is to say, a recombinant yeast suitable for use in a method of the invention may comprise a UGT which is capable of catalyzing a reaction in which steviol is converted to steviolmonoside.

[0089] Such a recombinant yeast suitable for use in a method of the invention may comprise a nucleotide sequence encoding a polypeptide having the activity shown by UDP-glycosyltransferase (UGT) UGT85C2, whereby the nucleotide sequence upon transformation of the yeast confers on that yeast the ability to convert steviol to steviolmonoside.

[0090] UGT85C2 activity is transfer of a glucose unit to the 13-OH of steviol. Thus, a suitable UGT85C2 may function as a uridine 5'-diphospho glucosyl: steviol 13-OH transferase, and a uridine 5'-diphospho glucosyl: steviol- 19-0- glucoside 13-OH transferase. A functional UGT85C2 polypeptides may also catalyze glucosyl transferase reactions that utilize steviol glycoside substrates other than steviol and steviol- 19-O-glucoside. Such sequences may be referred to as UGT1 sequences herein.

[0091] A recombinant yeast suitable for use in the invention may comprise a nucleotide sequence encoding a polypeptide which has UGT2 activity.

[0092] A polypeptide having UGT2 activity is one which functions as a uridine 5'-diphospho glucosyl: steviol- 13-O-glucoside transferase (also referred to as a steviol-13- monoglucoside 1,2-glucosylase), transferring a glucose moiety to the C-2' of the 13- O-glucose of the acceptor molecule, steviol- 13-O-glucoside. Typically, a suitable UGT2 polypeptide also functions as a uridine 5'-diphospho glucosyl: rubusoside transferase transferring a glucose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, rubusoside.

[0093] A polypeptide having UGT2 activity may also catalyze reactions that utilize steviol glycoside substrates other than steviol- 13-0-glucoside and rubusoside, e.g., functional UGT2 polypeptides may utilize stevioside as a substrate, transferring a glucose moiety to the C-2' of the 19-O-glucose residue to produce rebaudioside E. A functional UGT2 polypeptides may also utilize rebaudioside A as a substrate, transferring a glucose moiety to the C-2' of the 19-O-glucose residue to produce rebaudioside D. However, a functional UGT2 polypeptide may be one which does not transfer a glucose moiety to steviol compounds having a 1,3-bound glucose at the C- 13 position, i.e., transfer of a glucose moiety to steviol 1,3-bioside and 1,3-stevioside typically does not occur.

[0094] A polypeptide having UGT2 activity may also transfer sugar moieties from donors other than uridine diphosphate glucose. For example, a polypeptide having UGT2 activity act as a uridine 5'-diphospho D-xylosyl: steviol- 13 -O-glucoside transferase, transferring a xylose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, steviol- 13 -O-glucoside. As another example, a polypeptide having UGT2 activity may act as a uridine 5'-diphospho L-rhamnosyl: steviol- 13-0-glucoside transferase, transferring a rhamnose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, steviol.

[0095] A recombinant yeast suitable for use in the method of the invention may comprise a nucleotide sequence encoding a polypeptide having UGT activity may comprise a nucleotide sequence encoding a polypeptide capable of catalyzing the addition of a C-19-glucose to steviolbioside. That is to say, a recombinant yeast of the invention may comprise a UGT which is capable of catalyzing a reaction in which steviolbioside is converted to stevioside. Accordingly, such a recombinant yeast may be capable of converting steviolbioside to stevioside. Expression of such a nucleotide sequence may confer on the recombinant yeast the ability to produce at least stevioside.

[0096] A recombinant yeast suitable for use in a method of the invention may thus also comprise a nucleotide sequence encoding a polypeptide having the activity shown by UDP-glycosyltransferase (UGT) UGT74G1, whereby the nucleotide sequence upon transformation of the yeast confers on the cell the ability to convert steviolbioside to stevioside.

[0097] Suitable UGT74G1 polypeptides may be capable of transferring a glucose unit to the 13-OH and/or the 19-COOH of steviol. A suitable UGT74G1 polypeptide may function as a uridine 5'-diphospho glucosyl: steviol 19-COOH transferase and/or a uridine 5'-diphospho glucosyl: steviol- 13-O-glucoside 19-COOH transferase. Functional UGT74G1 polypeptides also may catalyze glycosyl transferase reactions that utilize steviol glycoside substrates other than steviol and steviol- 13-O-glucoside, or that transfer sugar moieties from donors other than uridine diphosphate glucose. Such sequences may be referred to herein as UGT3 sequences.

[0098] A recombinant yeast suitable for use in a method the invention may comprise a nucleotide sequence encoding a polypeptide capable of catalyzing glucosylation of the C-3' of the glucose at the C-13 position of stevioside. That is to say, a recombinant yeast suitable for use in a method of the invention may comprise a UGT which is capable of catalyzing a reaction in which stevioside is converted to rebaudioside A. Accordingly, such a recombinant yeast may be capable of converting stevioside to rebaudioside A. Expression of such a nucleotide sequence may confer on the yeast the ability to produce at least rebaudioside A.

[0099] A recombinant yeast suitable for use in a method of the invention may thus also comprise a nucleotide sequence encoding a polypeptide having the activity shown by UDP-glycosyltransferase (UGT) UGT76G1, whereby the nucleotide sequence upon transformation of a yeast confers on that yeast the ability to convert stevioside to rebaudioside A.

[0100] A suitable UGT76G1 adds a glucose moiety to the C-3' of the C-13-O-glucose of the acceptor molecule, a steviol 1,2 glycoside. Thus, UGT76G1 functions, for example, as a uridine 5'-diphospho glucosyl: steviol 13-0-1,2 glucoside C-3 ' glucosyl transferase and a uridine 5'-diphospho glucosyl: steviol- 19-0-glucose, 13-0-1,2 bioside C-3' glucosyl transferase. Functional UGT76G1 polypeptides may also catalyze glucosyl transferase reactions that utilize steviol glycoside substrates that contain sugars other than glucose, e.g., steviol rhamnosides and steviol xylosides. Such sequences may be referred to herein as UGT4 sequences. A UGT4 may alternatively or in addition be capable of converting RebD to RebM.

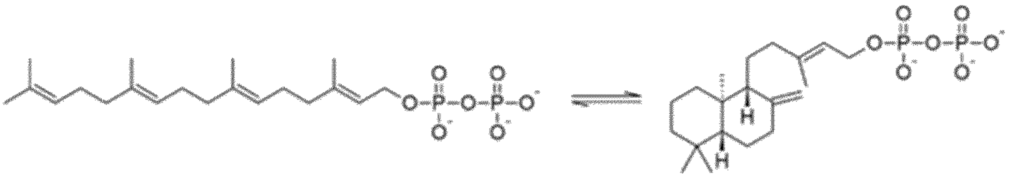
[0101] A recombinant yeast suitable for use in a method of the invention typically comprises nucleotide sequences encoding at least one polypeptide having UGT1 activity, at least one polypeptide having UGT2 activity, least one polypeptide having UGT3 activity and at least one polypeptide having UGT4 activity. One or more of these nucleic acid sequences may be recombinant. A given nucleic acid may encode a polypeptide having one or more of the above activities. For example, a nucleic acid encode for a polypeptide which has two, three or four of the activities set out above. Preferably, a recombinant yeast for use in the method of the invention comprises UGT1, UGT2 and UGT3 and UGT4 activity. Suitable UGT1, UGT2, UGT3 and UGT4 sequences are described in Table 1 of WO2015/007748.

[0102] A recombinant yeast host of the invention may comprise two or more nucleic acid sequences encoding a polypeptide having any one UGT activity, for example UGT1, 2, 3 or 4, activity. Where a recombinant host of the invention comprises two or more nucleic acid sequence encoding a polypeptide having any one UGT activity, those nucleic acid sequences may be the same or different and/or may encode the same or different polypeptides. In particular, a recombinant host of the invention may comprise a nucleic acid sequence encoding a two different UGT2 polypeptides.

[0103] A recombinant yeast host according to the invention may comprise one or more recombinant nucleotide sequence(s) encoding one of more of:

a polypeptide having ent-copalyl pyrophosphate synthase activity;
a polypeptide having ent-Kaurene synthase activity;
a polypeptide having ent-Kaurene oxidase activity; and
a polypeptide having kaurenoic acid 13-hydroxylase activity.

[0104] For the purposes of this invention, a polypeptide having *ent*-copalyl pyrophosphate synthase (EC 5.5.1.13) is capable of catalyzing the chemical reation:



[0105] This enzyme has one substrate, geranylgeranyl pyrophosphate, and one product, *ent*-copalyl pyrophosphate. This enzyme participates in gibberellin biosynthesis. This enzyme belongs to the family of isomerase, specifically the class of intramolecular lyases. The systematic name of this enzyme class is *ent*-copalyl-diphosphate lyase (decyclizing). Other names in common use include having *ent*-copalyl pyrophosphate synthase, *ent*-kaurene synthase A, and *ent*-kaurene synthetase A.

[0106] Suitable nucleic acid sequences encoding an ent-copalyl pyrophosphate synthase may for instance comprise a sequence as set out in SEQ ID. NO: 1, 3, 5, 7, 17, 19, 59, 61, 141, 142, 151, 152, 153, 154, 159, 160, 182 or 184 of WO2015/007748.

[0107] For the purposes of this invention, a polypeptide having ent-kaurene synthase activity (EC 4.2.3.19) is a polypeptide that is capable of catalyzing the chemical reaction:

ent-copalyl diphosphate ⇄ent-kaurene + diphosphate

[0108] Hence, this enzyme has one substrate, ent-copalyl diphosphate, and two products, ent-kaurene and diphosphate.

[0109] This enzyme belongs to the family of lyases, specifically those carbon-oxygen lyases acting on phosphates. The systematic name of this enzyme class is ent-copalyl-diphosphate diphosphate-lyase (cyclizing, ent-kaurene-forming). Other names in common use include ent-kaurene synthase B, ent-kaurene synthetase B, ent-copalyl-diphosphate diphosphate-lyase, and (cyclizing). This enzyme participates in diterpenoid biosynthesis.

[0110] Suitable nucleic acid sequences encoding an ent-Kaurene synthase may for instance comprise a sequence as set out in SEQ ID. NO: 9, 11, 13, 15, 17, 19, 63, 65, 143, 144, 155, 156, 157, 158, 159, 160, 183 or 184 of WO2015/007748.

[0111] *ent*-copalyl diphosphate synthases may also have a distinct *ent*-kaurene synthase activity associated with the same protein molecule. The reaction catalyzed by *ent*-kaurene synthase is the next step in the biosynthetic pathway to gibberellins. The two types of enzymic activity are distinct, and site-directed mutagenesis to suppress the *ent*-kaurene synthase activity of the protein leads to build up of *ent*-copalyl pyrophosphate.

[0112] Accordingly, a single nucleotide sequence used in a recombinant yeast host of the invention may encode a polypeptide having *ent*-copalyl pyrophosphate synthase activity and *ent*-kaurene synthase activity. Alternatively, the two activities may be encoded two distinct, separate nucleotide sequences.

[0113] For the purposes of this invention, a polypeptide having ent-kaurene oxidase activity (EC 1.14.13.78) is a polypeptide which is capable of catalysing three successive oxidations of the 4-methyl group of ent-kaurene to give kaurenoic acid. Such activity typically requires the presence of a cytochrome P450.

[0114] Suitable nucleic acid sequences encoding an ent-Kaurene oxidase may for instance comprise a sequence as set out in SEQ ID. NO: 21, 23, 25, 67, 85, 145, 161, 162, 163, 180 or 186 of WO2015/007748.

[0115] For the purposes of the invention, a polypeptide having kaurenoic acid 13-hydroxylase activity (EC 1.14.13) is one which is capable of catalyzing the formation of steviol (ent-kaur-16-en-13-ol-19-oic acid) using NADPH and O_2. Such activity may also be referred to as ent-ka 13-hydroxylase activity.

[0116] Suitable nucleic acid sequences encoding a kaurenoic acid 13-hydroxylase may for instance comprise a sequence as set out in SEQ ID. NO: 27, 29, 31, 33, 69, 89, 91, 93, 95, 97, 146, 164, 165, 166, 167 or 185 of WO2015/007748.

[0117] A recombinant yeast host of the invention may comprise a recombinant nucleic acid sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity. That is to say, a recombinant host of the invention may be capable of expressing a nucleotide sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity. For the purposes of the invention, a polypeptide having NADPH-Cytochrome P450 reductase activity (EC 1.6.2.4; also known as NADPH:ferrihemoprotein oxidoreductase, NADPH:hemoprotein oxidoreductase, NADPH:P450 oxidoreductase, P450 reductase, POR, CPR, CYPOR) is typically one which is a membrane-bound enzyme allowing electron transfer to cytochrome P450 in the microsome of the eukaryotic cell from a FAD- and FMN-containing enzyme NADPH:cytochrome P450 reductase (POR; EC 1.6.2.4).

[0118] In a recombinant yeast host of the invention, the ability of the host to produce geranylgeranyl diphosphate (GGPP) may be upregulated. Upregulated in the context of this invention implies that the recombinant host produces more GGPP than an equivalent non-recombinant host.

[0119] Accordingly, a recombinant yeast host of the invention may comprise one or more nucleotide sequence(s) encoding hydroxymethylglutaryl-CoA reductase, farnesyl-pyrophosphate synthetase and geranylgeranyl diphosphate synthase, whereby the nucleotide sequence(s) upon transformation of a host confer(s) on that host the ability to produce elevated levels of GGPP. Thus, a recombinant host according to the invention may comprise one or more recombinant nucleic acid sequence(s) encoding one or more of hydroxymethylglutaryl-CoA reductase, farnesyl-pyrophosphate synthetase and geranylgeranyl diphosphate synthase.

[0120] Accordingly, a recombinant yeast host of the invention may comprise nucleic acid sequences encoding one or more of:

a polypeptide having hydroxymethylglutaryl-CoA reductase activity;

a polypeptide having farnesyl-pyrophosphate synthetase activity.

A host of the present disclosure may be a prokaryotic archaebacterial or eukaryotic host cell.

[0121] A prokaryotic host cell may, but is not limited to, a bacterial host cell. An eukaryotic host cell may be, but is not limited to, a yeast, a fungus, an amoeba, an algae, an animal, an insect host cell.

[0122] An eukaryotic host cell may be a fungal host cell. "Fungi" include all species of the subdivision *Eumycotina* (Alexopoulos, C. J., 1962, In: Introductory Mycology, John Wiley & Sons, Inc., New York). The term fungus thus includes among others filamentous fungi and yeast.

[0123] "Filamentous fungi" are herein defined as eukaryotic microorganisms that include all filamentous forms of the subdivision *Eumycotina* and *Oomycota* (as defined by Hawksworth *et al.,* 1995, supra). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligatory aerobic. Filamentous fungal strains include, but are not limited to, strains of *Acremonium, Aspergillus, Agaricus, Aureobasidium, Cryptococcus, Corynascus, Chrysosporium, Filibasidium, Fusarium, Humicola, Magnaporthe, Monascus, Mucor, Myceliophthora, Mortierella, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Phanerochaete Podospora, Pycnoporus, Rhizopus, Schizophyllum, Sordaria, Talaromyces, Rasmsonia, Thermoascus, Thielavia, Tolypocladium, Trametes* and *Trichoderma.* Preferred filamentous fungal strains that may serve as host cells belong to the species *Aspergillus niger, Aspergillus oryzae, Aspergillus fumigatus, Penicillium chrysogenum, Penicillium citrinum, Acremonium chrysogenum, Trichoderma reesei, Rasamsonia emersonii* (formerly known as *Talaromyces emersonii*), *Aspergillus sojae, Chrysosporium lucknowense, Myceliophtora thermophyla.* Reference host cells for the comparison of fermentation characteristics of transformed and untransformed cells, include e.g. *Aspergillus niger* CBS120.49, CBS 513.88, *Aspergillus oryzae* ATCC16868, ATCC 20423, IFO 4177, ATCC 1011, ATCC 9576, ATCC14488-14491, ATCC 11601, ATCC12892, *Aspergillus fumigatus* AF293 (CBS101355), *P. chrysogenum* CBS 455.95, *Penicillium citrinum* ATCC 38065, *Penicillium chrysogenum* P2, *Acremonium chrysogenum* ATCC 36225, ATCC 48272, *Trichoderma reesei* ATCC 26921, ATCC 56765, ATCC 26921, *Aspergillus sojae* ATCC11906, *Chrysosporium lucknowense* ATCC44006 and derivatives of all of these strains. Particularly preferred as filamentous fungal host cell are *Aspergillus niger* CBS 513.88 and derivatives thereof.

[0124] An eukaryotic host cell may be a yeast cell. Preferred yeast host cells may be selected from the genera: *Saccharomyces* (e.g., *S. cerevisiae, S. bayanus, S. pastorianus, S. carlsbergensis), Brettanomyces, Kluyveromyces, Candida* (e.g., *C. krusei, C. revkaufi, C. pulcherrima, C. tropicalis, C. utilis), Issatchenkia* (eg. *I. orientalis) Pichia* (e.g., *P. pastoris* and *P. kudriavzevii*), *Schizosaccharomyces, Hansenula, Kloeckera, Pachysolen, Schwanniomyces, Trichosporon, Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica)), Yamadazyma*.

[0125] Prokaryotic host cells may be bacterial host cells. Bacterial host cell may be Gram negative or Gram positive bacteria. Examples of bacteria include, but are not limited to, bacteria belonging to the genus *Bacillus* (e.g., *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. puntis, B. megaterium, B. halodurans, B. pumilus,), Acinetobacter, Nocardia, Xanthobacter, Escherichia* (e.g., *E. coli* (e.g., strains DH 1 OB, Stbl2, DH5-alpha, DB3, DB3.1), DB4, DB5, JDP682 and ccdA-over (e.g., U.S. application No. 09/518,188))), *Streptomyces, Erwinia, Klebsiella, Serratia* (e.g., S. *marcessans), Pseudomonas* (e.g., *P. aeruginosa), Salmonella* (e.g., *S. typhimurium, S. typhi).* Bacteria also include, but are not limited to, photosynthetic bacteria (e.g., green non-sulfur bacteria (e.g., *Choroflexus* bacteria (e.g., *C. aurantiacus), Chloronema* (e.g., *C. gigateum*)), green sulfur bacteria (e.g., *Chlorobium bacteria* (e.g., *C. limicola), Pelodictyon* (e.g., *P. luteolum),* purple sulfur bacteria (e.g., *Chromatium* (e.g., *C. okenii*)), and purple non-sulfur bacteria (e.g., *Rhodospirillum* (e.g., *R. rubrum), Rhodobacter* (e.g. *R. sphaeroides, R. capsulatus),* and *Rhodomicrobium* bacteria (e.g., *R. anellii*)).

[0126] Host Cells may be host cells from non-microbial organisms. Examples of such cells, include, but are not limited to, insect cells (e.g., *Drosophila* (e.g., *D. melanogaster), Spodoptera* (e.g., *S. frugiperda* Sf9 or Sf21 cells) and *Trichoplusa* (e.g., High-Five cells); nematode cells (e.g., *C. elegans* cells); avian cells; amphibian cells (e.g., *Xenopus laevis* cells); reptilian cells; and mammalian cells (e.g., NIH3T3, 293, CHO, COS, VERO, C127, BHK, Per-C6, Bowes melanoma and HeLa cells).

[0127] A recombinant host according to the present disclosure may be able to grow on any suitable carbon source known in the art and convert it to a steviol glycoside. The recombinant host may be able to convert directly plant biomass, celluloses, hemicelluloses, pectines, rhamnose, galactose, fucose, maltose, maltodextrines, ribose, ribulose, or starch, starch derivatives, sucrose, lactose and glycerol. Hence, a preferred host expresses enzymes such as cellulases (endocellulases and exocellulases) and hemicellulases (e.g. endo- and exo-xylanases, arabinases) necessary for the conversion of cellulose into glucose monomers and hemicellulose into xylose and arabinose monomers, pectinases able to convert pectines into glucuronic acid and galacturonic acid or amylases to convert starch into glucose monomers. Preferably, the host is able to convert a carbon source selected from the group consisting of glucose, xylose, arabinose, sucrose, lactose and glycerol. The host cell may for instance be a eukaryotic host cell as described in WO03/062430, WO06/009434, EP1499708B1, WO2006096130 or WO04/099381.

[0128] Thus, in a further aspect, the invention also provides a process for the preparation of a steviol glycoside which comprises fermenting a recombinant yeast host of the invention which is capable of producing at least one steviol glycoside in a suitable fermentation medium, and optionally recovering the steviol glycoside.

[0129] The fermentation medium used in the process for the production of a steviol glycoside may be any suitable fermentation medium which allows growth of a particular eukaryotic host cell. The essential elements of the fermentation medium are known to the person skilled in the art and may be adapted to the host cell selected.

[0130] Preferably, the fermentation medium comprises a carbon source selected from the group consisting of plant biomass, celluloses, hemicelluloses, pectines, rhamnose, galactose, fucose, fructose, maltose, maltodextrines, ribose, ribulose, or starch, starch derivatives, sucrose, lactose, fatty acids, triglycerides and glycerol. Preferably, the fermentation medium also comprises a nitrogen source such as ureum, or an ammonium salt such as ammonium sulphate, ammonium chloride, ammoniumnitrate or ammonium phosphate.

[0131] The fermentation process according to the present invention may be carried out in batch, fed-batch or continuous mode. A separate hydrolysis and fermentation (SHF) process or a simultaneous saccharification and fermentation (SSF) process may also be applied. A combination of these fermentation process modes may also be possible for optimal productivity. A SSF process may be particularly attractive if starch, cellulose, hemicelluose or pectin is used as a carbon source in the fermentation process, where it may be necessary to add hydrolytic enzymes, such as cellulases, hemicellulases or pectinases to hydrolyse the substrate.

[0132] The recombinant yeast host used in the process for the preparation of a steviol glycoside may be any suitable recombinant host as defined herein above. It may be advantageous to use a recombinant eukaryotic recombinant host according to the invention in the process since most eukaryotic cells do not require sterile conditions for propagation and are insensitive to bacteriophage infections. In addition, eukaryotic host cells may be grown at low pH to prevent bacterial contamination.

[0133] The recombinant host according to the present disclosure may be a facultative anaerobic microorganism. A facultative anaerobic recombinant host can be propagated aerobically to a high cell concentration. This anaerobic phase can then be carried out at high cell density which reduces the fermentation volume required substantially, and may minimize the risk of contamination with aerobic microorganisms.

[0134] The fermentation process for the production of a steviol glycoside according to the present invention may be an aerobic or an anaerobic fermentation process.

[0135] An anaerobic fermentation process may be herein defined as a fermentation process run in the absence of oxygen or in which substantially no oxygen is consumed, preferably less than 5, 2.5 or 1 mmol/L/h, and wherein organic molecules serve as both electron donor and electron acceptors. The fermentation process according to the present invention may also first be run under aerobic conditions and subsequently under anaerobic conditions.

[0136] The fermentation process may also be run under oxygen-limited, or micro-aerobical, conditions. Alternatively, the fermentation process may first be run under aerobic conditions and subsequently under oxygen-limited conditions. An oxygen-limited fermentation process is a process in which the oxygen consumption is limited by the oxygen transfer from the gas to the liquid. The degree of oxygen limitation is determined by the amount and composition of the ingoing gasflow as well as the actual mixing/mass transfer properties of the fermentation equipment used.

[0137] The production of a steviol glycoside in the process according to the present invention may occur during the growth phase of the host cell, during the stationary (steady state) phase or during both phases. It may be possible to run the fermentation process at different temperatures.

[0138] The process for the production of a steviol glycoside may be run at a temperature which is optimal for the recombinant host. The optimum growth temperature may differ for each transformed recombinant host and is known to the person skilled in the art. The optimum temperature might be higher than optimal for wild type organisms to grow the organism efficiently under non-sterile conditions under minimal infection sensitivity and lowest cooling cost. Alternatively, the process may be carried out at a temperature which is not optimal for growth of the recombinant host.

[0139] The process for the production of a steviol glycoside according to the present invention may be carried out at any suitable pH value. If the recombinant host is a yeast, the pH in the fermentation medium preferably has a value of below 6, preferably below 5,5, preferably below 5, preferably below 4,5, preferably below 4, preferably below pH 3,5 or below pH 3,0, or below pH 2,5, preferably above pH 2. An advantage of carrying out the fermentation at these low pH values is that growth of contaminant bacteria in the fermentation medium may be prevented.

[0140] Such a process may be carried out on an industrial scale. The product of such a process is one or more steviol glycosides.

[0141] Recovery of steivol glycoside(s) from the fermentation medium may be performed by known methods in the art, for instance by distillation, vacuum extraction, solvent extraction, or evaporation.

[0142] In the process for the production of a steviol glycoside according to the invention, it may be possible to achieve a concentration of above 5 mg/l fermentation broth, preferably above 10 mg/l, preferably above 20 mg/l, preferably above 30 mg/l fermentation broth, preferably above 40 mg/l, more preferably above 50 mg/l, preferably above 60 mg/l, preferably above 70, preferably above 80 mg/l, preferably above 100 mg/l, preferably above 1 g/l, preferably above 5 g/l, preferably above 10 g/l, for example at least about 15g/L, such as at least about 20 g/l.

[0143] The present disclosure further provides a fermentation broth comprising a steviol glycoside obtainable by the process of the invention for the preparation of a steivol glycoside.

[0144] In the event that one or more steviol glycosides is expressed within a recombinant host of the invention, such cells may need to be treated so as to release them. Preferentially, at least one steviol glycoside, for example rebA or rebM, is produced extracellularly

[0145] The present disclosure also provides a steviol glycoside obtained by a process according to the invention for the preparation of a steviol glycoside or obtainable from a fermentation broth of the present disclosure. Such a steviol glycoside may be a non- naturally occurring steviol glycoside, that is to say one which is not produced in plants.

[0146] Also provided is a composition obtainable by a process of the invention (which typically comprises one or more steviol glycosides), Also provided is a composition comprising two or more steviol glycosides obtainable by a process of the invention for the preparation of a steviol glycoside or obtainable from a fermentation broth of the disclosure. In such a composition, one or more of the steviol glycosides may be a non- naturally occurring steviol glycoside, that is to say one which is not produced in plants. These are all compositions of the present disclosure.

[0147] A composition of the present disclosure may be used in any application known for such compounds. In particular, such a composition may for instance be used as a sweetener, for example in a food or a beverage. According to the present disclosure therefore, there is provided a foodstuff, feed or beverage which comprises a composition of the present disclosure.

[0148] For example a composition of the present disclosure may be formulated in soft drinks, as a tabletop sweetener, chewing gum, dairy product such as yoghurt (eg. plain yoghurt), cake, cereal or cereal-based food, nutraceutical, pharmaceutical, edible gel, confectionery product, cosmetic, toothpastes or other oral cavity composition, etc. In addition, a composition of the present disclosure can be used as a sweetener not only for drinks, foodstuffs, and other products dedicated for human consumption, but also in animal feed and fodder with improved characteristics.

[0149] Accordingly, the present disclosure provides, *inter alia,* a foodstuff, feed or beverage which comprises a composition of the present disclosure.

[0150] During the manufacturing of foodstuffs, drinks, pharmaceuticals, cosmetics, table top products, chewing gum the conventional methods such as mixing, kneading, dissolution, pickling, permeation, percolation, sprinkling, atomizing, infusing and other methods can be used.

[0151] A composition of the present disclosure can be used in dry or liquid forms. It can be added before or after heat treatment of food products. The amount of the sweetener depends on the purpose of usage. It can be added alone or in the combination with other compounds.

[0152] A composition of the present disclosure may be blended with one or more further non-caloric or caloric sweeteners. Such blending may be used to improve flavour or temporal profile or stability. A wide range of both non-caloric and caloric sweeteners may be suitable for blending with a composition of the present disclosure. For example, non-caloric sweeteners such as mogroside, monatin, aspartame, acesulfame salts, cyclamate, sucralose, saccharin salts or erythritol. Caloric sweeteners suitable for blending with a steviol glycoside or a composition of the present disclosure include sugar alcohols and carbohydrates such as sucrose, glucose, fructose and HFCS. Sweet tasting amino acids such as glycine, alanine or serine may also be used.

[0153] A composition of the present disclosure can be used in the combination with a sweetener suppressor, such as a natural sweetener suppressor. It may be combined with an umami taste enhancer, such as an amino acid or a salt thereof.

[0154] A composition of the present disclosure can be combined with a polyol or sugar alcohol, a carbohydrate, a physiologically active substance or functional ingredient (for example a carotenoid, dietary fiber, fatty acid, saponin, antioxidant, nutraceutical, flavonoid, isothiocyanate, phenol, plant sterol or stanol (phytosterols and phytostanols), a polyols, a prebiotic, a probiotic, a phytoestrogen, soy protein, sulfides/thiols, amino acids, a protein, a vitamin, a mineral, and/or a substance classified based on a health benefits, such as cardiovascular, cholesterol-reducing or antiinflammatory.

[0155] A composition of the present disclosure may include a flavoring agent, an aroma component, a nucleotide, an organic acid, an organic acid salt, an inorganic acid, a bitter compound, a protein or protein hydrolyzate, a surfactant, a flavonoid, an astringent compound, a vitamin, a dietary fiber, an antioxidant, a fatty acid and/or a salt.

[0156] A composition of the present disclosure may be applied as a high intensity sweetener to produce zero calorie, reduced calorie or diabetic beverages and food products with improved taste characteristics. Also it can be used in drinks, foodstuffs, pharmaceuticals, and other products in which sugar cannot be used.

[0157] In addition, a composition of the present disclosure may be used as a sweetener not only for drinks, foodstuffs, and other products dedicated for human consumption, but also in animal feed and fodder with improved characteristics.

[0158] The examples of products where a composition of the present disclosure can be used as a sweetening compound can be as alcoholic beverages such as vodka, wine, beer, liquor, sake, etc; natural juices, refreshing drinks, carbonated soft drinks, diet drinks, zero calorie drinks, reduced calorie drinks and foods, yogurt drinks, instant juices, instant coffee, powdered types of instant beverages, canned products, syrups, fermented soybean paste, soy sauce, vinegar, dressings, mayonnaise, ketchups, curry, soup, instant bouillon, powdered soy sauce, powdered vinegar, types of biscuits, rice biscuit, crackers, bread, chocolates, caramel, candy, chewing gum, jelly, pudding, preserved fruits and vegetables, fresh cream, jam, marmalade, flower paste, powdered milk, ice cream, sorbet, vegetables and fruits packed in bottles, canned and boiled beans, meat and foods boiled in sweetened sauce, agricultural vegetable food products, seafood, ham, sausage, fish ham, fish sausage, fish paste, deep fried fish products, dried seafood products, frozen food products, preserved seaweed, preserved meat, tobacco, medicinal products, and many others. In principal it can have unlimited applications.

[0159] The sweetened composition comprises a beverage, non-limiting examples of which include non-carbonated and carbonated beverages such as colas, ginger ales, root beers, ciders, fruit-flavored soft drinks (e.g., citrus-flavored soft drinks such as lemon-lime or orange), powdered soft drinks, and the like; fruit juices originating in fruits or vegetables, fruit juices including squeezed juices or the like, fruit juices containing fruit particles, fruit beverages, fruit juice beverages, beverages containing fruit juices, beverages with fruit flavorings, vegetable juices, juices containing vegetables, and mixed juices containing fruits and vegetables; sport drinks, energy drinks, near water and the like drinks (e.g., water with natural or synthetic flavorants); tea type or favorite type beverages such as coffee, cocoa, black tea, green tea, oolong tea and the like; beverages containing milk components such as milk beverages, coffee containing milk components, cafe au lait, milk tea, fruit milk beverages, drinkable yogurt, lactic acid bacteria beverages or the like; and dairy products.

[0160] Generally, the amount of sweetener present in a sweetened composition varies widely depending on the particular type of sweetened composition and its desired sweetness. Those of ordinary skill in the art can readily discern the appropriate amount of sweetener to put in the sweetened composition.

[0161] During the manufacturing of foodstuffs, drinks, pharmaceuticals, cosmetics, table top products, chewing gum the conventional methods such as mixing, kneading, dissolution, pickling, permeation, percolation, sprinkling, atomizing, infusing and other methods can be used.

[0162] Thus, compositions which incorporate a composition of the present disclosure can be made by any method known to those skilled in the art that provide homogenous even or homogeneous mixtures of the ingredients. These methods include dry blending, spray drying, agglomeration, wet granulation, compaction, co-crystallization and the like.

[0163] In solid form a composition of the present disclosure can be provided to consumers in any form suitable for delivery into the comestible to be sweetened, including sachets, packets, bulk bags or boxes, cubes, tablets, mists, or dissolvable strips. The composition can be delivered as a unit dose or in bulk form.

[0164] For liquid sweetener systems and compositions convenient ranges of fluid, semi-fluid, paste and cream forms, appropriate packing using appropriate packing material in any shape or form shall be invented which is convenient to carry or dispense or store or transport any combination containing any of the above sweetener products or combination of product produced above.

[0165] A composition of the present disclosure may include various bulking agents, functional ingredients, colorants, flavors.

[0166] The terms "sequence homology" or "sequence identity" or "homology" or "identity" are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percentage of sequence homology or sequence identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes. In order to optimize the alignment between the two sequences gaps may be introduced in any of the two sequences that are compared. Such alignment can be carried out over the full length of the sequences being compared. Alternatively, the alignment may be carried out over a shorter length, for example over about 20, about 50, about 100 or more nucleic acids/based or amino acids. The sequence identity is the percentage of identical matches between the two sequences over the reported aligned region.

[0167] A comparison of sequences and determination of percentage of sequence identity between two sequences can be accomplished using a mathematical algorithm. The skilled person will be aware of the fact that several different computer programs are available to align two sequences and determine the identity between two sequences (Kruskal, J. B. (1983) An overview of sequence comparison In D. Sankoff and J. B. Kruskal, (ed.), Time warps, string edits and macromolecules: the theory and practice of sequence comparison, pp. 1-44 Addison Wesley). The percent sequence identity between two amino acid sequences or between two nucleotide sequences may be determined using the Needleman and Wunsch algorithm for the alignment of two sequences. (Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). Both amino acid sequences and nucleotide sequences can be aligned by the algorithm. The Needleman-Wunsch algorithm has been implemented in the computer program NEEDLE. For the purpose of this invention the NEEDLE program from the EMBOSS package was used (version 2.8.0 or higher, EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice,P. Longden,I. and Bleasby,A. Trends in Genetics 16, (6) pp276-277, http://emboss.bioinformatics.nl/). For protein sequences EBLOSUM62 is used for the substitution matrix. For nucleotide sequence, EDNAFULL is used. The optional parameters used are a gap-open penalty of 10 and a gap extension penalty of 0.5. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

[0168] After alignment by the program NEEDLE as described above the percentage of sequence identity between a query sequence and a sequence of the invention is calculated as follows: Number of corresponding positions in the alignment showing an identical amino acid or identical nucleotide in both sequences divided by the total length of the alignment after subtraction of the total number of gaps in the alignment. The identity defined as herein can be obtained from NEEDLE by using the NOBRIEF option and is labeled in the output of the program as "longest-identity".

[0169] The nucleic acid and protein sequences can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17): 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See the homepage of the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/.

[0170] Standard genetic techniques, such as overexpression of enzymes in the host cells, genetic modification of host cells, or hybridisation techniques, are known methods in the art, such as described in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation, genetic modification etc of fungal host cells are known from e.g. EP-A-0 635 574, WO 98/46772, WO 99/60102 and WO 00/37671, WO90/14423, EP-A-0481008, EP-A-0635 574 and US 6,265,186.

[0171] A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

[0172] The present invention is further illustrated by the following Examples:

EXAMPLES

General

[0173] Standard genetic techniques, such as overexpression of enzymes in the host cells, as well as for additional genetic modification of host cells, are known methods in the art, such as described in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation and genetic modification of fungal host cells are known from e.g. EP-A-0 635 574, WO 98/46772, WO 99/60102 and WO 00/37671.

Example 1: Description of steviol glycoside production strain ML14094 (MAT-A lineage)

[0174] Two *Yarrowia lipolytica* strains of mating types MATA and MATB were engineered for steviol glycoside production. These strains were mated, the diploid sporulated, and spores with steviol glycoside production were selected. One of these spores was further developed for the production of steviol glycosides, including the production of Rebaudioside A.

[0175] Step 1: Strain ML10371 (MAT-A, lys1-, ura3-, leu2-) was transformed with 5 defined DNA fragments. All transformations were carried out via a lithium acetate/PEG fungal transformation protocol method and transformants were selected on minimal medium, YPD + 100 ug/ml nourseothricin or YPD + 100 ug/ml hygromycin, as appropriate.

1) a 7.0 kb DNA fragment isolated by gel purification following HindIII/NotI digestion of plasmid MB6969 (Figure 1). This construct encodes a synthetic construct for the overexpression of UGT2_1a (SEQ ID NO: 1) linked to the pPGM promoter (SEQ ID NO: 2) and xprT terminator (SEQ ID NO: 9) and the HPH hygromycin resistance gene, together flanked by lox sites (Güldener et al, 1996, Lambert et al, 2007), and a synthetic construct for the overexpression of the codon optimized *Y. lipolytica* hydroxymethylglutaryl-coenzyme A reductase open reading frame lacking the 5' membrane anchor sequence (tHMGopt: SEQ ID NO: 15) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10).

2) a 2.7 kb DNA fragment isolated by gel purification following HindIII/SspI digestion of MB6856 (Figure 2). This construct encodes tHMGopt (SEQ ID NO: 15) linked to the pHYPO promoter (SEQ ID NO: 4) and gpdT terminator (SEQ ID NO: 11).

3) a 2.5 kb DNA fragment isolated by gel purification following SspI digestion of MB6857 (Figure 3). This construct encodes tHMGopt (SEQ ID NO: 15) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID

NO: 10).

4) a 2.0 kb DNA fragment isolated by gel purification following SspI digestion of MB6948 (Figure 4). This construct encodes a synthetic construct for the overexpression of the codon optimized *Y. lipolytica* geranyl-geranyl-pyrophosphate synthetase (GGSopt: SEQ ID NO: 16) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10).

5) a 2.2 kb DNA fragment isolated by gel purification following HindIII/SspI digestion of MB6958 (Figure 5). This construct encodes GGSopt (SEQ ID NO: 16) linked to the pHYPO promoter (SEQ ID NO: 4) and gpdT terminator (SEQ ID NO: 11). The resulting strain was denoted ML13462.

[0176] Step 2. Strain ML13462 was transformed with a 9.7 kb fragment isolated by gel purification following SfiI digestion of plasmid MB7015 (Figure 6). This construct encodes a synthetic construct for the overexpression of UGT1 (SEQ ID NO: 17) linked to the pENO (SEQ ID NO: 5) promoter and gpdT terminator (SEQ ID NO: 11), UGT3 (SEQ ID NO: 18) linked to the pHSP promoter (SEQ ID NO: 3) and pgmT terminator (SEQ ID NO: 12), UGT4 (SEQ ID NO: 19) linked to the pCWP (SEQ NO: 6) promoter and pgkT terminator (SEQ ID NO: 13), and the lox-flanked nourseothricin resistance marker (NAT). Note that placement of lox sites allows for subsequent removal of nourseothricin resistance via CRE recombinase mediated recombination. A nourseothricin resistant isolate was denoted ML13500.

[0177] Step 3. Strain ML13500 was transformed with a 9.1 kb fragment isolated by gel purification following PvuI/SapI digestion of plasmid MB6986 (Figure 7). This construct encodes tHMGopt (SEQ ID NO: 15) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10), the lox-flanked URA3blaster prototrophic marker, and GGSopt (SEQ ID NO: 16) linked to the pHYPO promoter (SEQ ID NO: 4) and gpdT terminator (SEQ ID NO: 11). Transformants were selected on minimal medium lacking uracil. One selected uracil prototroph was denoted ML13723.

[0178] Step 4. Strain ML13723 was transformed with an 18.1 kb fragment isolated by gel purification following SfiI digestion of plasmid MB7059 (Figure 8). MB7059 encodes the tCPS_SR (SEQ ID NO: 20) linked to pCWP promoter (SEQ ID NO: 6) and cwpT terminator (SEQ ID NO: 10), the tKS_SR (SEQ ID NO: 21) linked to the pHYPO promoter (SEQ ID NO: 4) and gpdT terminator (SEQ ID NO: 11), the KAH_4 (SEQ ID NO: 22) linked to the pHSP promoter (SEQ ID NO: 3) and pgmT terminator (SEQ ID NO: 12), the KO_Gib (SEQ ID NO: 23) linked to the pTPI promoter (SEQ ID NO: 7) and pgkT terminator (SEQ ID NO: 13), the CPR_3 (SEQ ID NO: 24) linked to the pENO promoter (SEQ ID NO: 5) and xprT terminator (SEQ ID NO: 9) and the native *Y. lipolytica* LEU2 locus. One selected rebaudioside A-producing transformant was denoted ML14032.

[0179] Step 5. Strain ML14032 was struck to YPD and grown overnight and then struck to 5-FOA plates to allow for recombination mediated loss of the URA3 marker introduced previously. One selected 5-FOA resistant transformant was denoted ML14093.

[0180] Step 6. Strain ML14093 was transformed with a 19.0 kb fragment isolated by gel purification following SfiI digestion of plasmid MB7100 (Figure 9). MB7100 encodes the tCPS_SR (SEQ ID NO: 20) linked to the pHYPO promoter (SEQ ID NO: 4) and cwpT terminator (SEQ ID NO: 10), the tKS_SR (SEQ ID NO: 21) linked to the pCWP promoter (SEQ ID NO: 6) and gpdT terminator (SEQ ID NO: 11), the KAH_4 (SEQ ID NO: 22) linked to the pHSP promoter (SEQ ID NO: 3) and pgmT terminator (SEQ ID NO: 12), the KO_Gib (SEQ ID NO: 23) linked to the pENO promoter (SEQ ID NO: 5) and pgkT terminator (SEQ ID NO: 13), the CPR_3 (SEQ ID NO: 24) linked to the pTPI promoter (SEQ ID NO: 7) and xprT terminator (SEQ ID NO: 9) and URA3blaster prototrophic marker. Transformants were selected on minimal medium lacking uracil. One selected rebaudioside A producing uracil prototroph was denoted ML14094.

Example 2. Description of steviol glycoside production strain ML14087 (MAT-B lineage):

[0181]

Step 1. Strain ML13206 (MAT-B, ade1-, ure2-, leu2-) was transformed with 5 defined DNA fragments. All transformations were carried out via a lithium acetate/PEG fungal transformation protocol method and transformants were selected on minimal medium, YPD + 100 ug/ml nourseothricin or YPD + 100 ug/ml hygromycin, as appropriate.

1) a 7.0 kb DNA fragment isolated by gel purification following HindIII/NotI digestion of plasmid MB6969 (Figure 1). This construct encodes a synthetic construct for the overexpression of the codon pair optimized (CpO) ORF of UGT2_1a (SEQ ID NO: 1) linked to the pPGM (SEQ ID NO: 2) promoter and xprT terminator (SEQ ID NO: 9) and the HPH hygromycin resistance gene, together flanked by lox sites (Güldener et al, 1996, Lambert et al, 2007), and a synthetic construct for the overexpression of the codon optimized Y. lipolytica hydroxymethylglutaryl-coenzyme A reductase open reading frame lacking the 5' membrane anchor sequence (tHMGopt: SEQ ID NO: 15) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10).

2) a 2.7 kb DNA fragment isolated by gel purification following HindIII/SspI digestion of MB6856 (Figure 2). This construct encodes tHMGopt (SEQ ID NO: 15) linked to the pHYPO promoter (SEQ ID NO: 4) and gpdT terminator

(SEQ ID NO: 11).

3) a 2.5 kb DNA fragment isolated by gel purification following SspI digestion of MB6857 (Figure 3). This construct encodes tHMGopt (SEQ ID NO: 15) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10).

4) a 2.0 kb DNA fragment isolated by gel purification following SspI digestion of MB6948 (Figure 4). This construct encodes a synthetic construct for the overexpression of the codon optimized Y. lipolytica geranyl-geranyl-pyrophosphate synthetase (GGSopt: SEQ ID NO: 16) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10).

5) a 2.2 kb DNA fragment isolated by gel purification following HindIII/SspI digestion of MB6958 (Figure 5). This construct encodes GGSopt (SEQ ID NO: 16) linked to the pHYPO (SEQ ID NO: 4) promoter and gpdT terminator (SEQ ID NO: 11). The resulting strain was denoted ML13465.

Step 2. Strain ML13465 was transformed with 2 defined DNA fragments:

1). a 9.7 kb fragment isolated by gel purification following SfiI digestion of plasmid MB7015 (Figure 6). This construct encodes a synthetic construct for the overexpression of UGT1 (SEQ ID NO: 17) linked to the pENO promoter (SEQ ID NO: 5) and gpdT (SEQ ID NO: 11) terminator, UGT3 (SEQ ID NO: 18) linked to the pHSP promoter (SEQ ID NO: 3) and pgmT terminator (SEQ ID NO: 12), UGT4 (SEQ ID NO: 19) linked to the pCWP promoter (SEQ ID NO: 6) and pgkT terminator (SEQ ID NO: 13), and the lox-flanked nourseothricin resistance marker (NAT). Note that placement of lox sites allows for subsequent removal of nourseothricin resistance via CRE recombinase mediated recombination.

2). a 9.1 kb fragment isolated by gel purification following PvuI/SapI digestion of plasmid MB6988 (Figure 10). This construct encodes tHMGopt (SEQ ID NO: 15) linked to the pHSP promoter (SEQ ID NO: 3) and cwpT terminator (SEQ ID NO: 10), the lox-flanked URA2blaster prototrophic marker, and GGSopt (SEQ ID NO: 16) linked to the pHYPO promoter (SEQ ID NO: 4) and gpdT terminator (SEQ ID NO: 11). Strains were selected on YPD + 100 ug/ml nourseothricin and replica plated to minimal medium lacking uracil. A nourseothricin resistant, uracil prototrophic isolate was denoted ML13490

Step 3. Strain ML13490 was struck to YPD and grown overnight and then struck to 5-FOA plates to allow for recombination mediated loss of the URA2 marker introduced previously. One selected 5-FOA resistant transformant was denoted ML13501.

Step 4. Strain ML13501 was transformed with a 9.1 kb fragment isolated by gel purification following PvuI/SapI digestion of plasmid MB6988 (Figure 10). Transformants were selected on minimal medium lacking uracil. One selected uracil prototroph was denoted ML13724.

Step 5. Strain ML13724 was transformed with an 18.1 kb fragment isolated by gel purification following SfiI digestion of plasmid MB7044 (Figure 11). MB7044 encodes the tCPS_SR (SEQ ID NO: 20) linked to the pHYPO promoter (SEQ ID NO: 4) and cwpT terminator (SEQ ID NO: 10), the tKS_SR (SEQ ID NO: 21) linked to the pCWP promoter (SEQ ID NO: 6) and gpdT terminator (SEQ ID NO: 11), the KAH_4 (SEQ ID NO: 22) linked to the pHSP promoter (SEQ ID NO: 3) and pgmT terminator (SEQ ID NO: 12), the KO_Gib (SEQ ID NO: 23) linked to the pENO promoter (SEQ ID NO: 5) and pgkT terminator (SEQ ID NO: 13), the CPR_3 (SEQ ID NO: 24) linked to the pTPI promoter (SEQ ID NO: 7) and xprT terminator (SEQ ID NO: 9) and the LEU2 locus. One selected rebaudioside A-producing transformant was denoted ML14044.

Step 6. Strain ML14044 was struck to YPD and grown overnight and then struck to 5-FOA plates to allow for recombination mediated loss of the URA2 marker introduced previously. One selected 5'-FOA resistant transformant was denoted ML14076.

Step 7. Strain ML14076 was transformed with a 19.0 kb fragment isolated by gel purification following SfiI digestion of plasmid MB7094 (Figure 12). MB7094 encodes the tCPS_SR (SEQ ID NO: 20) linked to the pHYPO promoter (SEQ ID NO: 4) and cwpT terminator (SEQ ID NO: 10), the tKS_SR (SEQ ID NO: 21) linked to the pCWP promoter (SEQ ID NO: 6) and gpdT terminator (SEQ ID NO: 11), the KAH_4 (SEQ ID NO: 22) linked to the pHSP promoter (SEQ ID NO: 3) and pgmT terminator (SEQ ID NO: 12), the KO_Gib (SEQ ID NO: 23) linked to the pENO promoter (SEQ ID NO: 5) and pgkT terminator (SEQ ID NO: 13), the CPR_3 (SEQ ID NO: 24) linked to the pTPI promoter (SEQ ID NO: 7) and xprT terminator (SEQ ID NO: 9) and URA2blaster prototrophic marker. Transformants were selected on minimal medium lacking uracil. One selected rebaudioside A producing uracil prototroph was denoted ML14087.

Example 3. Mating MATA and MATB lineage and selecting steviol glycoside-producing progeny

[0182] Strains of opposite mating types (ML14094 and ML14087) with complementary nutritional deficiencies (ADE1+ lys1- and ade1- LYS1+) were allowed to mate and then plated on selective media that would allow only diploids to grow (minimal media lacking both adenine and lysine). Diploid cells (ML14143) were then induced to undergo meiosis and sporulation by starvation, and the resulting haploid progenies were replica-plated to identify prototrophic isolates with hygromycin and nourseothricin resistance. One selected rebaudioside A-producing strain was denoted ML14737

Example 4. Making the strain UGT2 1a-free

[0183] The hygromycin antibiotic marker and the nourseothricin antibiotic marker were removed from strain ML14737 after transformation with MB6128 (Figure 13) which encodes a construct for constitutive overexpression of the CRE recombinase. CRE recombinase deletes the antibiotics markers by recombination over the Lox66 and Lox71 sites. An inactive Lox72 site is left in the genome (Güldener et al, 1996, Lambert et al, 2007). Plasmid MB6128 is a CEN plasmid which replicates episomally in Yarrowia lipolytica and which contains the CRE recombinase coding region under control of the native Yarrowia lipolytica pHHF promoter and hhfT terminator, and a neoR (encoding for G418 resistance) under the control of the native Yarrowia lipolytica pTEF1 promoter and xprT terminator. After selection of MB6128 transformants on YPD + G418 and screening for transformants that lost hygromycin and nourseothricin resistance by successful Cre-Lox recombination, the sensitive colonies were grown on non-selective medium to remove the MB6128 CEN plasmid (spontaneous loss of the CEN plasmid). The resulting antibiotic marker-free variant is denoted ML14869. This strain no longer produces rebaudioside A due to the loss of UGT2_1a along with the hygromycin resistance and produces the intermediate rubusoside instead.

Example 5. Introduction of UGT2 10b

[0184] ML14869 was transformed with a 4.2 kb DNA fragment produced by PCR and purified following gel electrophoresis. The fragment encoded a sequence optimized variant of UGT2_10b (SEQ ID NO: 25) and hygromycin resistance. The DNA fragment was generated by fusion PCR as follows. UGT2_10b was codon pair optimized for expression in *Y. lipolytica* and synthesized by DNA2.0, linked to the native *Yarrowia lipolytica* pHSP promoter and gpdT terminator and flanked by connector sequences. This 1.4 kb DNA fragment was amplified using appropriate oligos and purified by gel electrophoresis. The HPH marker was flanked by lox sites, and linked to the *Ashbya gossypii* pTEF1 promoter and tef1T terminator and flanked by connector sequences. This 1.8 kb DNA fragment was amplified using appropriate oligos and purified by gel electrophoresis. A 4.2 kb DNA fragment was obtained by PCR using these two DNA fragments with followed by gel electrophoresis and purification. Transformation of ML14869 with this defined DNA fragment and selection on YPD + 100 ug/ml hygromycin yielded the rebaudioside A producing strain ML14937.

Example 6. Making strain ML14937 marker-free

[0185] The hygromycin antibiotic marker was removed from strain ML14937 after transformation with MB6128 (Figure 13) which encodes a construct for constitutive overexpression of the CRE recombinase. CRE recombinase deletes the antibiotics markers by recombination over the Lox66 and Lox71 sites. An inactive Lox72 site is left in the genome (Güldener et al, 1996, Lambert et al, 2007). Plasmid MB6128 is a CEN plasmid which replicates episomally in *Yarrowia lipolytica* and which contains the CRE recombinase coding region under control of the native *Yarrowia lipolytica* pHHF promoter and hhfT terminator and a neoR (encoding for G418 resistance) under the control of the native *Yarrowia lipolytica* pTEF1 promoter and xprT terminator. After selection of MB6128 transformants on YPD + G418 and screening for transformants that lost hygromycin and nourseothricin resistance by successful Cre-Lox recombination, the sensitive colonies were grown on non-selective medium to remove the MB6128 CEN plasmid (spontaneous loss of the CEN plasmid). The resulting antibiotic marker-free variant is denoted ML14958.

Example 7. Transformation with extra gene copies

[0186] Strain ML14958 was struck to YPD and grown overnight and then struck to 5-FOA plates to allow for recombination-mediated loss of the URA2 marker. One selected 5'-FOA resistant transformant was denoted ML15075. Strain ML15075 was transformed with 3 defined DNA fragments and selected for transformation on YPD with 100 ug/ml hygromycin. The three fragments were as follows:

1) a 4.6 kb DNA fragment encoding the KAH open reading frame linked to the native Y. *lipolytica* pHYPO promoter and the xprT terminator and also encoding the HPH hygromycin resistance gene flanked by lox sites, produced by

PCR and purified following gel electrophoresis. Sequences were assembled in *Saccharomyces cerevisiae,* and DNA from this S. *cerevisiae* strain was used as template for PCR yielding the 4.6 kb DNA fragment (see Figure 14) used to transform ML15075.

2) a 3.3 kb DNA fragment encoding the tCPS open reading frame linked to the native *Y. lipolytica* pHSP promoter and xprT terminator, produced by PCR and purified following gel electrophoresis. Sequences were assembled in *Saccharomyces cerevisiae,* and DNA from this *S. cerevisiae* strain was used as template for PCR yielding the 3.3 kb DNA fragment (Figure 15) used to transform ML15075.

3) a 9.1 kb fragment isolated by gel purification following PvuI/SapI digestion of plasmid MB6986 (Figure 16). This construct encodes tHMG linked to the native Y. lipolytica HSP promoter and CWP terminator, the lox-flanked URA3blaster prototrophic marker, and GGS1 linked to the native Y. lipolytica HYPO promoter and GPD terminator. ML15075 is auxotrophic due to a mutation in ura2, so this fragment was not selected for.

[0187] One selected hygromycin-resistant transformant was denoted ML15085.

Example 8. Transformation of extra copies of tHMG and GGS

[0188] Strain ML15085 was transformed with a 8.4 kb fragment isolated by gel purification following PvuI/SapI digestion of plasmid MB6988 (Figure 10). This construct encodes tHMGopt linked to the native *Y. lipolytica* pHSP promoter and cwpT terminator, the lox-flanked URA2blaster prototrophic marker, and GGSopt linked to the native *Y. lipolytica* pHYPO promoter and gpdT terminator. Transformants were selected on minimal medium lacking uracil. One selected uracil prototroph was denoted ML15086.

Example 9. Making strain ML15086 marker-free

[0189] The hygromycin antibiotic marker was removed from strain ML15086 after transformation with MB6128 (Figure 13) which encodes a construct for constitutive overexpression of the CRE recombinase. CRE recombinase deletes the antibiotics markers by recombination over the Lox66 and Lox71 sites. An inactive Lox72 site is left in the genome (Güldener et al, 1996, Lambert et al, 2007). Plasmid MB6128 is a CEN plasmid which replicates episomally in *Yarrowia lipolytica* and which contains the CRE recombinase coding region under control of the native *Yarrowia lipolytica* pHHF promoter and hhfT terminator and a neoR (encoding for G418 resistance) under the control of the native *Yarrowia lipolytica* pTEF1 promoter and xprT terminator.

[0190] After selection of MB6128 transformants on YPD + G418 and screening for transformants that lost hygromycin and nourseothricin resistance by successful Cre-Lox recombination, the sensitive colonies were grown on non-selective medium to remove the MB6128 CEN plasmid (spontaneous loss of the CEN plasmid). One prototrophic, antibiotic marker-free variant is denoted ML15087.

Example 10. Disruption of YALI0C08701 in ***Y. lipolytica* ML15087**

[0191] To increase the efficiency of targeted transporter disruptions and avoid integration events at other loci in the genome than targeted for, YALI0C08701 (SEQ ID NO: 26), an important factor in non-homologous end joining, was disrupted. Disruption constructs were designed based on single cross-over integration using internal homologous fragments to target the disruption construct to the YALI0C08701 ORF. The internal homologous fragments used to assemble the disruption constructs were PCR amplified from *Y. lipolytica* genomic DNA using suitable primers which were elongated with appropriate connector sequences. The total length of the PCR fragments was 600bp. 500bp of these fragments are homologous to the targeted YALI0C08701 and 50 bp to the vector backbone and KanMX marker cassette. The KanMX marker cassette was PCR amplified with suitable primers. For both flanks and marker cassette six 50 μl PCR reactions were perfomed using Phusion polymerase (New England Biolabs) according to suppliers' instructions. The PCR products were purified and concentrated using NucleoSpin Gel and PCR Clean-up Kit (Machery Nagel).

[0192] The flanks and marker were assembled in the SnaBI/PmeI digested pRS417 5_3 (Figure 17) shuttle vector backbone in-vivo by transforming both flanks, the KanMX fragment and the linear pRS417 5_3 shuttle vector to S. cerevisiae CEN.PK113-7D. After transformation and recovery for 2 hours in YEPhD at 30°C the cells were plated on YEPhD agar with 200 μg/ml G418 (Invitrogen). The plates were incubated at 30°C for 2 days. Transformants were cultured in YEPD + 200 μg/ml G418 at 30°C. Plasmid DNA was isolated and purified.

[0193] Correct assembly of the disruption cassettes was established with diagnostic PCR. The expression cassettes were PCR amplified in six 50 μl PCR reactions. The PCR product was purified and concentrated using NucleoSpin Gel and PCR Clean-up Kit (Machery Nagel). 1 μg of the PCR amplified disruption cassette was transformed to Y. lipolytica strain ML15087. After transformation and recovery for 2 hours in YEPhD at 30°C the cells were plated on YEPhD agar with 400 μg/ml G418. The plates were incubated at 30°C for 2 days. Transformants were purified by re-streaking them on YEPhD agar with 400 μg/ml G418. One of the transformants was named STV2049. Correct integration was established with diagnostic PCR using appropriate oligo's.

Example 11. Disruption of transporter YALI0E25201 in ***Y. lipolytica*** **STV2049**

[0194] Disruption constructs were designed based on single cross-over integration using internal homologues fragments to target the disruption construct to the YALI0E25201 ORF (SEQ ID NO: 27). The internal homologous fragments used to assemble the disruption constructs were ordered as synthetic DNA in the form of gBlocks (IDT) with a total length of 700bp. 600bp of these fragments are homologous to the targeted transporter YALI0E25201 and 50 bp to the vector backbone (5 and 3 connector sequence, Figure 18) and HygB marker cassette (c and d connector sequence, Figure 18). The HygB marker cassette was PCR amplified with suitable primers using Phusion polymerase (New England Biolabs) according to suppliers' instructions. The PCR product was purified and concentrated using NucleoSpin Gel and PCR Clean-up Kit (Machery Nagel).

[0195] The flanks and marker were assembled in the SnaBI/PmeI digested pRS417 5_3 shuttle vector backbone in-vivo by transforming both flanks, the HygB fragment and the linear pRS417 5_3 shuttle vector to *S. cerevisiae* CEN-PK-7D. See Figure 18.

[0196] After transformation and recovery for 2 hours in YEPhD at 30°C the cells were plated on YEPhD agar with 200 μg/ml G418 (Invitrogen). The plates were incubated at 30°C for 2 days. Transformants were cultured in YEPD + 200 μg/ml G418 at 30°C, 550 rpm and 80% humidity. Plasmid DNA was isolated and purified. Correct assembly of the disruption cassettes was established with diagnostic PCR.

[0197] The expression cassettes were PCR amplified (Figure 19) in six 50 μl PCR reactions. The PCR product was purified and concentrated using NucleoSpin Gel and PCR Clean-up Kit (Machery Nagel). 1 μg of the PCR amplified disruption cassette was transformed to *Y. lipolytica* STV2049. After transformation and recovery for 2 hours in YEPhD at 30°C the cells were plated on YEPhD agar with 100 μg/ml HygB (Invitrogen). The plates were incubated at 30°C for 2 days. Transformants were purified by re-streaking them on YEPhD agar with 100 μg/ml HygB. Correct integration, as illustrated in Figure 20, was established with diagnostic PCR using appropriate oligo's.

Example 12. Fermentation of ***Y. lipolytica*** **STV2049 and STV2049 YALI0E25201 disruption transformants**

[0198] A pre-culture was inoculated with colony material from YEPh-D agar. The pre-culture was grown in 96-Half Deep Well Plate containing 200 μl 0.5xYEP with 2% glucose per well. The plates were sealed with a breathable seal and incubated in an Infors incubator at 30°C, 80% humidity, 750 rpm for 48 hours.

[0199] 40 μl of the 96-well pre-culture was used to inoculate a 24-well deep well plate containing 2.5 ml of 0.25x YEP with 5% glucose per well. Plates were sealed with a breathable seal and incubated in an Infors incubator at 30°C, 80% humidity, 500 rpm for 120 hours.

[0200] The 24-well plates were spun down in an MTP centrifuge and 1 ml of the supernatant was harvested. The remaining supernatant was decanted from the pellet.

[0201] The supernatant fraction was diluted 1000 times in 33% Acetonitrile. The pellet was suspended in 2.5ml milli-Q and 1 ml was transferred to a 96-well DWP. The plate was sealed with an aluminium seal and incubated for 10 minutes at 90°C. The plate was cooled down to room temperature and 0.5 ml of 100% Acetonitrile was added and homogenized. The plates were centrifuged at 2088 xg for 10 minutes to pellet cell material and debris. The supernatant of the pellet fraction was diluted 33 times in 33% acetonitrile resulting in a combined 50 times dilution. Samples were analyzed for Rebaudioside A and other steviolglycosides using LC/MS.

[0202] We found that the strains that had the YALI0E25201 disruption produced lower titers of Rebaudioside A in the supernatant compared to the parent strain. The concentration of Rebaudioside A was approximately three fold lower in the transporter disruption strain compared to the parental strain (see Table 1).

Table 1. Rebaudioside A supernatant concentrations in 24-well fermentations. Six replicate cultures were performed for parent strain STV2049. Duplicate cultures of three independent STV2049 ΔYALI0E25201 transformants were performed. The averages are taken for the data in Table 1.

Strain	RebA supernatant (mg/L)
STV2049	441
STV2049 ΔYALI0E25201 A	155

[0203] The observation that the concentration of Rebaudioside A in the supernatant is lower for the transporter disruption strain compared to the reference strain was also seen for Stevioside, Rubusoside, and to a lesser degree for Rebaudioside D and Steviol-19-monoside (see Tables 2 to 5).

Table 2. Stevioside supernatant concentrations in 24-well fermentations. Six replicate cultures were performed for parent strain STV2049. Duplicate cultures of three independent STV2049 ΔYALI0E25201 transformants were performed. The averages are taken for the data in Table 2.

Strain	Stevioside supernatant (mg/L)
STV2049	144
STV2049 ΔYALI0E25201 A	46.9

Table 3. Rubusoside supernatant concentrations in 24-well fermentations. Six replicate cultures were performed for parent strain STV2049. Duplicate cultures of three independent STV2049 ΔYALI0E25201 transformants were performed. The averages are taken for the data in Table 3.

strain	Rubusoside supernatant (mg/L)
STV2049	42.2
STV2049 ΔYALI0E25201 A	17.2

Table 4. Rebaudioside D supernatant concentrations in 24-well fermentations. Six replicate cultures were performed for parent strain STV2049. Duplicate cultures of three independent STV2049 ΔYALI0E25201 transformants were performed. The averages are taken for the data in Table 4.

Strain	RebD supernatant (mg/L)
STV2049	39.7
STV2049 ΔYALI0E25201 A	32.6

Table 5. Steviol-19-monoside supernatant concentrations in 24-well fermentations. Six replicate cultures were performed for parent strain STV2049. Duplicate cultures of three independent STV2049 ΔYALI0E25201 transformants were performed. The averages are taken for the data in Table 5.

Strain	Steviol-19-monoside supernatant (mg/L)
STV2049	35.7
STV2049 ΔYALI0E25201 A	20.2

[0204] The effect of disrupting the transporter gene was most pronounced on the transport of the aforementioned steviol glycosides, and not a consequence of a general decreased production of steviol glycosides. This is illustrated when the concentration of all steviol glycosides are measured in the pellet fraction (Table 6). Here it can be seen that in the YALI0E25201 disruption strain, the concentration of all steviol glycosides in the pellet fraction is increased in the transporter disruption strain, indicative of reduced transport.

Table 6. Concentration of the sum of all steviol glycosides (Rebaudioside A. Stevioside, Rebaudioside B, Rebaudioside D, Steviolbioside, Rubusoside, Steviol-19-monoside, Steviol-13-monoside and Rebaudioside M) in the pellet fraction in 24-well fermentations. Six replicate cultures were performed for parent strain STV2049. Duplicate cultures of three independent STV2049 ΔYALI0E25201 transformants were performed. The averages are taken for the data in Table 6.

strain	Sum steviol glycosides pellet (uM)
STV2049	34
STV2049 ΔYALI0E25201 A	67

Example 13. Over-expression of the YALI0E25201 transporter in steviol glycosides producing *Y. lipolytica* strains

[0205] To further demonstrate the functionality of the YALI0E25201 transporter, the YALI0E25201 ORF was assembled in an expression cassette with the *Y. lipolytica* YP006 promoter and *Y. lipolytica* TEF4 terminator. The cassettes were assembled in the pRS417 5_3 vector together with the Nourseothricin marker. As a negative control the same cassette only containing the Nourseothricin marker was constructed. The expression cassettes were PCR amplified and the obtained fragments were transformed to three different strains: strains STV2049 is a strain producing mostly RebA, and is described above. Also, the transporter deletion strain is included (STV2049 ΔYALI0E25201 (described above)). The third strain is STV2170, a strain producing mostly RebM. STV2170 was build similarly to strain STV2049, and the genotype is listed below in Table 7.

Table 7. Genotype of strain STV2170. Between brackets indicates the gene copy number present in the strain

Strain name	genotype
STV2170	tHMG (2; SEQ ID NO: 15) GGS (2; SEQ ID NO: 16) CarG (1; SEQ ID NO: 32) CPS (2 SEQ ID NO: 20) KS (2; SEQ ID NO: 21) KO_Gib (2; SEQ ID NO: 23), KAH4 (4; SEQ ID NO: 22) CPR3 (2; SEQ ID NO: 24) UGT1 (5; SEQ ID NO: 17) UGT2_6b (2; SEQ ID NO: 33) UGT3 (2; SEQ ID NO: 18) UGT4 (4; SEQ ID NO: 19) RT18 (1; SEQ ID NO:34)

[0206] Six transformants were selected for each combination of strain and expression cassette. The transformants were grown in 24-well fermentation and the supernatant- and pellet fractions were analyzed by LC-MS as described in Example 12.

Table 8. RebA supernatant and pellet concentrations in 24-well fermentations in the control strain (STV2049 with NatMX marker) and YALI0E25201 transporter over-expression strain (STV2049 with transporter and NatMX marker)

Strain	RebA supernatant (mg/L)	RebA pellet (mg/L)
STV2049 control	392	15
YALI0E25201 O.E.	461	15

[0207] These data illustrate that over-expression of the YALI0E25201 transporter has a positive effect on extracellular RebA production.

Table 9. RebM supernatant and pellet concentrations in 24-well fermentations in the control strain (STV2049 with NatMX marker) and YALI0E25201 transporter over-expression strain (STV2049 with transporter and NatMX marker)

Strain	RebM supernatant (mg/L)	RebM pellet (mg/L)
STV2049 control	59	10
YALI0E25201 O.E.	43	4

[0208] RebM production in this strain is low compared to RebA production, but even so, the effect of the transporter over-expression can be seen in the concentrations of RebM. As RebA is more efficiently exported to outside the cell in the YALI0E25201 over-expression strain, less RebA will be available for further glycosylation inside the cell, and hence resulting in lower production of RebM, particularly in the pellet fraction.

Table 10. RebA supernatant and pellet concentrations in 24-well fermentations in the transporter deletion strain (STV2049 ΔYALI0E25201 with NatMX marker) and the same background with the YALI0E25201 transporter over-expressed (STV2049 ΔYALI0E25201 with transporter and NatMX marker.)

Strain	RebA supernatant (mg/L)	RebA pellet (mg/L)
STV2049 ΔYALI0E25201 control	114	23
STV2049 ΔYALI0E25201 YALI0E25201 O.E.	431	17

[0209] Upon over-expression of the YALI0E25201 transporter in the YALI0E25201 deletion strain, the extracellular production of RebA is greatly enhanced, and restored to similar levels as the reference strain without the transporter deletion.

Table 11. RebM supernatant and pellet concentrations in 24-well fermentations in the transporter deletion strain (STV2049 ΔYALI0E25201 with NatMX marker) and the same background with the YALI0E25201 transporter over-expressed (STV2049 ΔYALI0E25201 YALI0E25201 O.E.)

Strain	RebM supernatant (mg/L)	RebM pellet (mg/L)
STV2049 ΔYALI0E25201 control	6	45
STV2049 ΔYALI0E25201 YALI0E25201 O.E.	22	1

[0210] In the transporter deletion strain, steviol glycosides including RebA accumulate in the cell, allowing for continued glycosylation inside the cell. As a consequence, RebM concentrations may increase. In the transporter deletion strain, the concentration RebM in the pellet fraction is much higher than in the supernatant. Upon restoring transport this is reversed: less accumulation of intracellular RebM, and more export of RebM.

Table 12. RebA supernatant and pellet concentrations in 24-well fermentations in the RebM production control strain (STV2170 with NatMX marker) and YALI0E25201 transporter over-expression strain (STV2170 with transporter and NatMX marker)

Strain	RebA supernatant (mg/L)	RebA pellet (mg/L)
STV2170 control	107	22
STV2170 YALI0E25201 O.E.	283	8

[0211] Over-expression of the YALI0E25201 transporter results in greatly increased extracellular production of RebA, and greatly reduced accumulation of RebA in the pellet.

Table 13. RebM supernatant and pellet concentrations in 24-well fermentations in the RebM production control strain (STV2170 with NatMX marker) and YALI0E25201 transporter over-expression strain (STV2170 with transporter and NatMX marker)

Strain	RebM supernatant (mg/L)	RebM pellet (mg/L)
STV2170 control	631	132
STV2170 YALI0E25201 O.E.	660	61

[0212] Over-expression of the YALI0E25201 transporter results in increased extracellular production of RebM, and reduced accumulation of RebM in the pellet.

[0213] Together these data illustrate that over-expression of the YALI0E25201 transporter has a positive effect on extracellular RebA and RebM production. Not only is the distribution of RebA and RebM production in the supernatant fraction versus the pellet fraction favourable when the transporter is over-expressed, over-expression of the YALI0E25201 transporter also has a positive effect on the total amount of RebA and RebM production.

Table 14 Description of the sequence listing

SEQ ID NO	**Description**	**SEQ ID NO**	**Description**
SEQ ID NO: 1	UGT2_1a CpO for *Y. lipolytica*	SEQ ID NO: 18	UGT3 CpO for *Y. lipolytica*
SEQ ID NO: 2	PGM promoter from *Y. lipolytica*	SEQ ID NO: 19	UGT4 CpO for *Y. lipolytica*
SEQ ID NO: 3	HSP promoter from *Y. lipolytica*	SEQ ID NO: 20	tCPS from S. rebaudiana CpO for *Y. lipolytica*
SEQ ID NO: 4	HYPO promoter from *Y. lipolytica*	SEQ ID NO: 21	tKS from S. rebaudiana CpO for *Y. lipolítica*

(continued)

SEQ ID NO	Description	SEQ ID NO	Description
SEQ ID NO: 5	ENO promoter from *Y. lipolytica*	SEQ ID NO: 22	KAH_4 CpO for *Y. lipolítica*
SEQ ID NO: 6	CWP promoter from *Y. lipolytica*	SEQ ID NO: 23	KO from *Gibberella fujikori* CpO for *Y. lipolytica*
SEQ ID NO: 7	TPI promoter from *Y. lipolytica*	SEQ ID NO: 24	CPR_3 CpO for *Y. lipolytica*
SEQ ID NO: 8	YP001 promoter from *Y. lipolytica*	SEQ ID NO: 25	UGT2_10b CpO for *Y. lipolytica*
SEQ ID NO: 9	Xpr terminator from *Y. lipolytica*	SEQ ID NO: 26	YALI0C08701 WT CDS
SEQ ID NO: 10	Cwp terminator from *Y. lipolytica*	SEQ ID NO: 27	YALI0E25201 WT CDS
SEQ ID NO: 11	Gpd terminator from *Y. lipolytica*	SEQ ID NO: 28	YALI0E25201 CpO for *Y. lipolytica*
SEQ ID NO: 12	Pgm terminator from *Y. lipolytica*	SEQ ID NO: 29	YALI0E25201 WT from *Y. lipolytica*
SEQ ID NO: 13	Pgk terminator from *Y. lipolytica*	SEQ ID NO: 30	YP006 promoter from *Y. lipolytica*
SEQ ID NO: 14	act1T terminator from *Y. lipolytica*	SEQ ID NO: 31	Tef4 terminator from *Y. lipolytica*
SEQ ID NO: 15	tHMG CpO for *Y. lipolítica*	SEQ ID NO: 32	CarG codon optimized for *Y. lipolytica*
SEQ ID NO: 16	GGS CpO for *Y. lipolytica*	SEQ ID NO: 33	UGT2_6b CpO for *Y. lipolytica*
SEQ ID NO: 17	UGT1 CpO for *Y. lipolytica*	SEQ ID NO: 34	RT18 CpO for *Y. lipolytica*

SEQUENCE LISTING

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aagatctaca acgacaccaa ggtcgagaag gagtacgttt cccagtttgt cgactacctc 1380

gagaagaacg cccgagctgt cgccattgac cacgagagtt aa 1422

<210> 2
<211> 803
<212> DNA
<213> Yarrowia lipolytica

<400> 2

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aaatcaaggc gtgaaccact gtttgaggac aaatgtgaca caaccaacca gtgtcagggg 180

caagtccgtg acaaagggga agatacaatg caattactga cagttacgga ctgcctcgat 240

gccctaacct tgccccaaaa taagacaact gtcctcgttt aagcgcaacc ctattcagcg 300

tcacgtcata atagcgtttg gatagcacta gtctatgagg agcgttttat gttgcggtga 360

gggcgattgg tgctcatatg ggttcaattg aggtggtgga acgagcttag tcttcaattg 420

aggtgcgagc gacacaattg ggtgtcacgt ggcctaattg acctcggatc gtggagtccc 480

cagttataca gcaaccacga ggtgcatgag taggagacgt caccagacaa tagggttttt 540

ttggactgga gagggtaggg caaaagcgct caacgggctg tttggggagc tatgggggag 600

gaattggcga tatttgtgag gttgacggct ccgatttgcg tgttttgtcg cttctgcatc 660

tccccatacc catatcttcc ctccccacct ctttccacga taattttacg gatcagcaat 720

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<210> 3
<211> 995
<212> DNA
<213> Yarrowia lipolytica

<400> 3

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gacgtccaaa ccgtccaaag tggcagggtg acgtgatgcg acgcacgaag gagatggccc 180

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atttcacatt cgccctcaat ggccactttg cggtggtgaa catggtttct gaaacaaccc 360 cccagaatta gagtatattg atgtgtttaa gattgggttg ctatttggcc attgtggggg 420

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<210> 4
<211> 1004
<212> DNA
<213> Yarrowia lipolytica

<400> 4

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<210> 5
<211> 1422
<212> DNA
<213> Yarrowia lipolytica

<400> 5

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ccaagcaaac atgagtatgt gcttgtatgt actgaaaccc gtctacggta atattttaga       540
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cgaggtaaga cacattcggt ggttcaggcg gctacttgta tgtagcatcc acgttcatgt       660
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atagcgcaat tgggtttgca tcatgtataa aaggagcaat cccccactag ttataaagtc      1380
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<210> 6
<211> 865
<212> DNA
<213> Yarrowia lipolytica

<400> 6

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gaatgcgaaa gatggcaata ttggtggagg aggcgacggc ggtaaacaat ggagatagag     180

accacaaaag agacctggag acccaaaatg gactcacgac aactccccca ctcccccact     240

ccccatctcc ccctgggcat cagttgccca tcggtatctc aactgtcgca ctagttagcg     300

caaccatcac atactttaga cgccaaacaa tgggacaact catcgcgccg aactatgggc     360

agattttaac tcgcacaaca ttaccccaac tctaaaaggt aacctcgacc ggaaaacggg     420

aagacaggat cagcaaccgt gatcgacaga atcttcaggg cactacagtt gatagacata     480

ggttatgttg gtaggtctag acgggcctcg gggaattgac cccaccagtt gcaagtcacg     540

tgcccctgat acagctagtt tagcacatct gcccactacg tctggacgca ccatggtggt     600

gccagtcgcg tgaactcaaa cacccactag cctcgggaag gattcagtta aatccgcacc     660

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acattccagg actctgttca aggtcaaata tacaaaacac agatagagaa acatagacag     780

ctgcggcctt ataaatacct gggcgcactt ctctcttttt ccctcctcat cacacattcg     840

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<210> 7
<211> 880
<212> DNA
<213> Yarrowia lipolytica

<400> 7

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actatttctt tacaggtgac tcagaaagtg catgttggaa atgagccaca gaccaagaca 180

agatatgaca aaattgcact attcgatgca gaattcgacg gtgtttccat tggtgttatg 240

acattcatct gcattcatac aaaaaagtct tggtagtggt acttttgcgt tattacctcc 300

gatatctacg caccccccaa cccccctgct acagtaaaga gtgtgagtct actgtacatg 360

cttactaaac cacctactgt acagcgaaac ccctcagcaa aatcacacaa tcagctcatt 420

acaacacacc caatgacctc accacaaatt ctatacgcct tttgacgcca ttattacagt 480

agcttgcaac gccgttgtct taggttccat ttttagtgct ctattacctc acttaacccg 540

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gttagggcat ccgataatat cgcctggact cggcgccata ttctcgactt ctgggcgcgt 720

tgtattcatc tcctccgctt cccaacactt ccacccgttt ctccatccca accaatagaa 780

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tctccttcgt actcgtacat acaacacaac tacattcaaa 880

<210> 8
<211> 1000
<212> DNA
<213> Yarrowia lipolytica

<400> 8

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cactcttacc cggctccacc ttctagaagc caccaccgat ccaccgacga tgatcagtcc 180

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caaccattgc tcctccggct acacctgctc aaaggacttg ttccacactc ttccccagct 840

ctcccacgca aacaaagaga gcaaccttaa gtggacagct catgagcact cccctcgttt 900

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<210> 9
<211> 127
<212> DNA
<213> Yarrowia lipolytica

<400> 9

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gatttatgta acgaaactga aatttgacca gatattgttg taaatagaaa atctggcttg 120

taggtgg 127

<210> 10
<211> 200
<212> DNA
<213> Yarrowia lipolytica

<400> 10

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tatagccatt gtaacaaaag ccggctatcg accgctttat cgaagaatat ttcccgcccc 180

ccagtggcca aacgatatcg 200

<210> 11
<211> 200
<212> DNA
<213> Yarrowia lipolytica

<400> 11

ctatccgaag atcaagagcg aagcaagttg taagtccagg acatgtttcc cgcccacgcg 60

agtgatttat aacacctctc ttttttgaca cccgctcgcc ttgaaattca tgtcacataa 120

attatagtca acgacgtttg aataacttgt cttgtagttc gatgatgatc atatgattac 180

attaatagta attactgtat 200

<210> 12
<211> 200
<212> DNA
<213> Yarrowia lipolytica

<400> 12

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gtagctattg tgattgtgtg attttcgtct tgctggttct tattgtgtcc cattcgtttc 180

atcctgatga ggacccctgg 200

<210> 13
<211> 200
<212> DNA
<213> Yarrowia lipolytica

<400> 13

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agaaaatttc agtgaagttg tgttttctat atagtaagca cttttggtac aagtatctgc 120

acatccctgc atgttacaag cctgatcatg cagggcaata ttctgactat aaatatacct 180

cgatatttta gcaagctata 200

<210> 14
<211> 300
<212> DNA
<213> Yarrowia lipolytica

<400> 14

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gctgtgtact ccattcattg aagggtgtag tcgtgtttta ttgcatgagc gcctattact       180

cgtataagta actgttttgt aacacttcat gaacggagat ggtatgaaca gaagtaataa       240

tatcctggaa gtcagctgtg cccagaggtg tgtgtgggtg tggcatactt tgggacaaca       300
```

<210> 15
<211> 1503
<212> DNA
<213> Artificial sequence

<220>
<223> tHMG CpO for Yarrowia lipolitica

<400> 15

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aagcccgtga ccgagacccg ttctctggac gacttggagg ctatcatgaa ggcaggtaag     180
accaagctcc tggaggacca cgaggttgtc aagctctctc tcgaaggcaa gctccctttg     240
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tag                                                                  1503
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<210> 16
<211> 984
<212> DNA
<213> Artificial sequence

<220>
<223> GGS CpO for Yarrowia lipolitica

<400> 16

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aagtactttg aggatgcgca gtga 984

<210> 17
<211> 1446
<212> DNA
<213> Artificial sequence

<220>
<223> UGT1 CpO for Yarrowia lipolitica

<400> 17

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tgtctggacg gtgctcccgg tttccgattt gagactatcc ccgatggtgt ctcccactcc 240
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aactaa 1446

<210> 18
<211> 1383
<212> DNA
<213> Artificial sequence

<220>
<223> UGT3 CpO for Yarrowia lipolitica

<400> 18

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cagggccaca tcaacccctt catccagttc ggcaagcgac tcatctccaa gggtgtcaag 120

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<223> UGT4 CpO for Yarrowia lipolitica

<400> 19

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<400> 20

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attgtgattt aa 2232

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<223> tKS-SR CpO for Yarrowia lipolitica

<400> 21

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<223> KAH_4 CpO for Yarrowia lipolitica

<400> 22

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<223> KO_Gib CpO for Yarrowia lipolitica

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<210> 24
<211> 2133

<212> DNA
<213> Artificial sequence

<220>
<223> CPR_3 CpO for Yarrowia lipolitica

<400> 24

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<220>
<223> UGT2_10b CpO for Y. lipolitica

<400> 25

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<213> Yarrowia lipolytica

<400> 27

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Glu Arg Ala Gly Ile Leu Ser Lys Leu Thr Trp Gln Trp Leu Ser Pro
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Leu Leu Lys Thr Gly Tyr Leu Arg Asn Ile Glu Arg Glu Asp Leu Tyr
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Lys Val Arg Glu Arg Asn Ser Ala Ala Val Ile Gln Gln Arg Leu Glu
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Ser Asn Leu Glu Lys Gln Tyr Ala Lys Tyr His Ala Lys Leu Leu Lys
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Lys Gly Leu Ser Glu Gln Glu Ala His Leu Lys Leu Gln Asp Ser Ala
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Tyr Arg Gly Thr Asp Arg Glu Pro Lys Val Gly Arg Gly Val Gly Pro
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Ser Ile Gly Leu Phe Leu Leu Ala Val Gly Val Thr Phe Phe Phe Asn
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His Met Phe Tyr Asn Val Lys Met Val Gly Ala Gln Ala Arg Ala Ala
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Arg Ala Gln Tyr Thr Thr Gly Lys Ile Thr Asn Leu Ala Ala Ile Asp
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Phe Leu Pro Val Val Gly Cys Ala Ile Ala Val Leu Val Val Asn Leu
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Lys Val Ala Ala Leu Val Gly Ile Ala Thr Met Ile Val Leu Ile Phe
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Val Lys Leu Thr Asp Lys Arg Val Thr Tyr Ile Arg Glu Ala Leu Gln
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Lys Ile Lys Lys Val Arg Leu Asp Glu Thr Arg Asn Tyr Ala Lys Met
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Leu Pro Ala Leu Ala Val Leu Pro Gln Ala Leu Gln Ala Ala Gly Asp
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Ser Thr Pro Thr Thr Val Phe Asp Ala Thr Leu Glu Ser Thr Asp Asp
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Ala Lys Glu Ile Ala Val Val Val Glu Glu Glu Val Glu His Glu Lys
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Thr Glu Trp Ile Gly Cys Leu Glu Pro Trp Ile Gln Asn Ala Thr Val
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Arg Asp Asn Ile Val Phe Gly Arg Lys Phe Asp Ser Glu Trp Tyr Arg
    610                 615                 620
```

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Thr Val Val Thr Ala Cys Gln Leu Ser Gln Asp Leu Lys Ile Met Thr
625                 630                 635                 640

His Gly Asp Asn Thr Met Ile Gly Glu Arg Gly Ile Thr Val Ser Gly
                645                 650                 655

Gly Gln Lys Ala Arg Ile Asn Leu Ala Arg Ala Ile Tyr Gly Asn Pro
            660                 665                 670

Glu Ile Leu Ile Met Asp Asp Val Leu Ser Ala Val Asp Ala Arg Val
        675                 680                 685

Gly Ala Gly Ile Val Asp Asp Cys Leu Arg Gly Leu Ala Lys Asn Ser
    690                 695                 700

Thr Arg Ile Leu Ala Thr His Gln Leu Ser Val Leu Pro Lys Ala Asp
705                 710                 715                 720

His Val Ile Phe Met Asp Ala Glu Gly Gln Phe His Ile Gly Thr Tyr
                725                 730                 735

Gln Glu Leu Glu Ala Asp Asn Glu Gln Phe Lys Ala Leu Leu Ala Ala
            740                 745                 750

Gly Ser Met Ser Lys Glu Glu Val Val Ala Val Asp Glu Thr Glu Val
        755                 760                 765

Val Ile Glu Gly Asp Leu Glu Asp Asp Cys Asp Asn Lys Glu Glu Tyr
    770                 775                 780

Glu Asp Ala Ala Glu Thr Ile Ser Ile Leu Ala Asp Ala Thr Gln Glu
785                 790                 795                 800

Leu Gln Lys Val Thr Thr Thr Val Ser Ala Phe Glu Glu Asn Asp Asn
                805                 810                 815

Met Met Glu Glu Glu Glu Arg Met Arg Asp Ala Val Gly Leu His Val
            820                 825                 830

Tyr Trp Gln Tyr Phe Arg Gln Ala Asn Pro Ser Arg Val Lys Val Met
        835                 840                 845

Met Phe Ile Gly Met Ile Phe Ile Ser Met Ile Val Ile Ala Phe Leu
    850                 855                 860

Phe Val Phe Thr Ser Val Trp Leu Ser Phe Trp Thr Gly Asp Arg Phe
865                 870                 875                 880
```

```
His Ala Ser Arg Asn Phe Tyr Thr Gly Ile Tyr Ile Met Leu Gly Ile
                885                 890                 895

Leu Leu Leu Leu Ala Val Ala Gly Tyr Met Ile Val Asn Glu Ile Asn
            900                 905                 910

Ser Ala Met Ala Ala Arg Asn Leu His Asn His Ala Leu Asp Ser Val
        915                 920                 925

Phe Ala Ala Arg Thr Ser Phe Phe Asp Thr Thr Pro Gln Gly Arg Ile
    930                 935                 940

Ile Asn Arg Phe Thr Arg Asp Thr Asp Ser Leu Asp Asn Glu Leu Ala
945                 950                 955                 960

Met Arg Leu Thr Met Leu Phe Phe Gly Val Ser Ala Phe Phe Ser Asn
                965                 970                 975

Phe Leu Leu Thr Cys Val Tyr Val Pro Tyr Val Thr Leu Val Leu Val
            980                 985                 990

Pro Val Gly Phe Val Phe Tyr Val  Ser Leu Gly Tyr Tyr  Arg Lys Ser
        995                 1000                 1005

Ala Arg  Glu Val Lys Arg Ile  Asp Ser Ile Glu Arg  Ser His Met
    1010                 1015                 1020

Met Ser  Val Phe Asn Glu Ser  Ile Ser Gly Met Pro  Val Ile Ile
    1025                 1030                 1035

Met Tyr  Lys Ala Gln His Arg  Leu Met Asn Lys Leu  Gln Ala Thr
    1040                 1045                 1050

Leu Asp  Asp Met Asp Ser Ala  Tyr Phe Leu Thr Ala  Ala Asn Gln
    1055                 1060                 1065

Arg Trp  Leu Ser Leu Arg Leu  Asp Gly Leu Gly Ser  Leu Val Val
    1070                 1075                 1080

Leu Val  Ala Thr Ile Leu Val  Ala Val Gly Val Phe  Asp Leu Thr
    1085                 1090                 1095

Pro Ser  Asn Met Gly Leu Ile  Ile Ser Ala Ala Ser  Phe Ile Pro
    1100                 1105                 1110

Glu Val  Met Ser Met Val Ala  Gln Ala Val Ala Glu  Leu Glu Asn
```

```
        1115                1120                1125

Cys Met  Asn Ala Thr Glu Arg  Ile Leu Tyr Tyr Lys  Asp Asn Ile
    1130                1135                1140

Pro Ala  Glu Ala Ala Arg Glu  Val Asp Gly Thr Glu  Leu Asp Gln
    1145                1150                1155

Arg Pro  Asn Trp Pro Glu Gln  Gly Ala Ile Ser Phe  Asn Asn Val
    1160                1165                1170

Ser Met  Lys Tyr Arg Asp Gly  Leu Pro Tyr Val Leu  Lys Ser Leu
    1175                1180                1185

Ser Val  Asp Phe Gln Gly Gly  His Lys Val Gly Ile  Cys Gly Arg
    1190                1195                1200

Thr Gly  Ala Gly Lys Ser Thr  Ile Leu Gln Thr Leu  Tyr Arg Ile
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Val Glu  Leu Ala Glu Gly Ser  Ile Thr Ile Asp Gly  Val Asp Ile
    1220                1225                1230

Ser Thr  Ile Gly Leu His Gln  Leu Arg Ser Gln Leu  Ser Ile Ile
    1235                1240                1245

Pro Gln  Glu Pro Val Leu Phe  Leu Gly Thr Ile Arg  Ser Asn Leu
    1250                1255                1260

Asp Pro  Leu Glu Gln Tyr Ser  Asp Ala Glu Leu Trp  Gly Ser Leu
    1265                1270                1275

Arg Arg  Ser Gly Leu Leu Asp  Glu Gly Glu Thr Glu  Gly Lys Phe
    1280                1285                1290

His Leu  Asp Gln Lys Val Glu  Ala Asp Gly Ser Asn  Phe Ser Leu
    1295                1300                1305

Gly Glu  Arg Gln Leu Leu Thr  Leu Ala Arg Ala Leu  Leu Arg Asn
    1310                1315                1320

Thr Lys  Ile Leu Val Leu Asp  Glu Ala Thr Ser Asn  Val Asp Tyr
    1325                1330                1335

Lys Thr  Asp Lys Leu Val Gln  Glu Thr Ile Ser Arg  Glu Phe Gly
    1340                1345                1350
```

```
His Cys Thr Ile Leu Cys Ile Ala His Arg Leu Arg Thr Ile Ala
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Lys Tyr Asp Arg Ile Leu Val Leu Glu Ser Gly Glu Ile Asn Gln
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Tyr Asp Thr Pro Trp Asn Leu Tyr Asn Asp Lys Glu Gly Ile Phe
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Arg Gly Met Cys Asp Thr Ser Gly Leu Asn Glu Val Asp Phe Asn
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Lys
```

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<213> Yarrowia lipolytica

<400> 30

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ttcttcttcc agacctaccc gttcacacaa ctacaagttg ccgccttaaa caacaacgtg     180
gtcaactccg gagttaacag aagcataata atgtgatgga atttggaggt tggggagaga     240
cagtttggac cggagacacg ccacggggaa atcatcataa acattggtaa aatgccaaaa     300
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cagttttttt tcttttcttt cggcaaaata accttcgcag tcatgtgaga tatcgcacga     660
caagatgtga ctaacatgcc aacggcggct gcccccaagg tgtatatgag taccaaatta     720
gggcatgata caagaatacc tttcgaaaag ccggaacaag ataaagcagc ccaaccctta     780
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```

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<400> 31

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tactgtaatt gcatgtccca ccttctttgc accgttcttg ttgtatacag tacaatacat 180

acataccccta tgtatgtttt ttgtgaatat gatgagtcta ctactacagt aaatcagctt 240

tgatccctgc cagaatgtgt gtacacagta tgggactctc atcccctgta caatataata 300

<210> 32
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<213> Artificial sequence

<220>
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gacctctggc tgcacgttcc taaggatgtt ctttgcgtca tcaacaagat tatcggtatg 180

ctgcacaacg cctctcttat gattgacgat gttcaggacg actctgatct ccgacgagga 240

gtccccgttg ctcaccacat ttacggtgtc cctcagacta ttaacaccgc taactacgtg 300

attttcctcg cccttcagga ggttatgaag ctgaacatcc cttctatgat gcaggtgtgt 360

accgaggagc ttattaacct ccaccgaggt cagggaattg agctgtactg gcgagattcc 420

ctcacttgtc ccactgagga ggagtacatt gatatggtta acaacaagac ctctggcctc 480

cttcgacttg ccgtccgact gatgcaggct gcttctgagt ccgacatcga ctacacccct 540

ctcgtcaaca ttatcggaat tcacttccag gttcgagatg actacatgaa cctccagtcc 600

acctcttaca ctaacaacaa gggcttttgc gaggacctga ccgagggaaa gttctccttc 660

cctattattc acgctattcg aaaggacccc tctaaccgac agctcctgaa cattatctct 720

cagaagccca cctccattga ggttaagaag tacgctcttg aggtgatccg aaaggctgga 780

tcttttgagt acgttcgaga gttccttcga cagaaggagg ctgagtccct gaaggagatc 840

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tctcctctca tcaacgttgt ccagctcacc ctcccccgag tccaggagct gcccgaggac 240

gccgaggcca ccaccgatgt ccacccccgag gatatcccct acctcaagaa ggcctccgac 300

ggtctgcagc ccgaggtcac cgagttcctc gagcagcact ctcccgactg gatcatctac 360

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tccaagacct accacgagtt tggcacccag tggctccgac tcctcgagac tctccaccga 720

aagcccgtca tccccgtcgg tctgctccct ccctccatcc ccggctccga caaggacgac 780

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gagatccccc gaaacgaaga ggacggttcc ttcacccgag actctgttgc tgagtctctc 1260

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<400> 34

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tgccgaaact ccaataaata a                                               1341
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Claims

1. A recombinant host capable of producing a steviol glycoside which overexpresses a nucleic acid which encodes a polypeptide which mediates steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least 70% sequence identity thereto, and wherein the amount of total steviol glycoside outside the cell as compared to inside the cell is higher as compared to a corresponding cell which does not overexpress a nucleic acid which encodes a polypeptide which mediates steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least 70% sequence identity thereto, wherein the recombinant host is a yeast.

2. A recombinant host according to claim 1 wherein the host belongs to one of the genera *Saccharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Issatchenkia, Brettanomyces, Pachysolen* and *Yarrowia,* preferably wherein the recombinant host is a *Saccharomyces cerevisiae* cell, a *Yarrowia lipolytica* cell, a *Candida krusei* cell or an *Issatchenkia orientalis* cell.

3. A recombinant Yarrowia host capable of producing a steviol glycoside, which has been modified, preferably in its genome, to result in a deficiency in the production of a polypeptide which mediates steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least 70% sequence identity thereto, and wherein the amount of total steviol glycoside outside the cell as compared to inside the cell is lower as compared to a corresponding cell which does not have a deficiency in the production of a polypeptide which mediates steviol glycoside transport and which polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29 or an amino acid sequence having at least 70% sequence identity thereto, wherein deficiency in the production of said polypeptide mediating steviol glycoside transport is generated by modifying, partially deleting or entirely deleting a nucleotide sequence encoding the polypeptide such that substantially no expression of the polypeptide takes place in the host cell.

4. A recombinant host according to any one of the preceding claims which comprises one or more recombinant nucleotide sequence(s) encoding:

a polypeptide having ent-copalyl pyrophosphate synthase activity;
a polypeptide having ent-Kaurene synthase activity;
a polypeptide having ent-Kaurene oxidase activity; and
a polypeptide having kaurenoic acid 13-hydroxylase activity.

5. A recombinant host according to any one of the preceding claims, which comprises a recombinant nucleic acid sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity.

6. A recombinant host according to any one of the preceding claims which comprises a recombinant nucleic acid sequence encoding one or more of:

(i) a polypeptide having UGT74G1 activity;
(ii) a polypeptide having UGT2 activity;
(iii) a polypeptide having UGT85C2 activity; and
(iv) a polypeptide having UGT76G1 activity.

7. A recombinant host according to any one of claims 3 to 6, wherein the recombinant host is a *Yarrowia lipolytica.*

8. A recombinant host according to any one of the preceding claims, wherein the ability of the host to produce geranylgeranyl diphosphate (GGPP) is upregulated.

9. A recombinant host according to any one of the preceding claims which comprises a nucleic acid sequence encoding one or more of:

a polypeptide having hydroxymethylglutaryl-CoA reductase activity; or
a polypeptide having farnesyl-pyrophosphate synthetase activity.

10. A process for the preparation of a steviol glycoside which comprises fermenting a recombinant host according to any one of the preceding claims in a suitable fermentation medium and, optionally, recovering the steviol glycoside.

11. A process according to claim 10 for the preparation of a steviol glycoside, wherein the process is carried out on an industrial scale.

Patentansprüche

1. Rekombinanter Wirt, in der Lage, ein Steviolglycosid zu erzeugen, welcher eine Nukleinsäure überexprimiert, die für ein Polypeptid kodiert, das Steviolglycosidtransport vermittelt, und wobei das Polypeptid die in SEQ ID NO: 29 angegebene Aminosäuresequenz oder eine Aminosäuresequenz mit mindestens 70 % Sequenzidentität dazu umfasst, und wobei die Menge an Gesamtsteviolglycosid außerhalb der Zelle im Vergleich zu innerhalb der Zelle höher ist als bei einer entsprechenden Zelle, welche nicht eine Nukleinsäure überexprimiert, die für ein Polypeptid kodiert, das Steviolglycosidtransport vermittelt, und wobei das Polypeptid die in SEQ ID NO: 29 angegebene Aminosäuresequenz oder eine Aminosäuresequenz mit mindestens 70 % Sequenzidentität dazu umfasst, wobei der rekombinante Wird eine Hefe ist.

2. Rekombinanter Wirt nach Anspruch 1, wobei der Wirt zu einer der Gattungen *Saccharomyces,* Pichia, *Kluyveromyces, Candida, Hansenula, Issatchenkia, Brettanomyces, Pachysolen* und *Yarrowia* gehört, vorzugsweise, wobei der rekombinante Wirt eine Zelle von *Saccharomyces cerevisiae,* eine Zelle von *Yarrowia lipolytica,* eine Zelle von *Candida krusei* oder eine Zelle von *Issatchenkia orientalis* ist.

3. Rekombinanter Yarrowia-Wirt, in der Lage, ein Steviolglycosid zu erzeugen, der modifiziert worden ist, vorzugsweise in seinem Genom, um zu einem Mangel an der Produktion eines Polypeptids zu führen, das Steviolglycosidtransport vermittelt, und wobei das Polypeptid die in SEQ ID NO: 29 angegebene Aminosäuresequenz oder eine Aminosäuresequenz mit mindestens 70 % Sequenzidentität dazu umfasst, und wobei die Menge an Gesamtsteviolglycosid außerhalb der Zelle im Vergleich zu innerhalb der Zelle niedriger ist als bei einer entsprechenden Zelle, welche nicht einen Mangel an der Produktion eines Polypeptids aufweist, das Steviolglycosidtransport vermittelt, und wobei das Polypeptid die in SEQ ID NO: 29 angegebene Aminosäuresequenz oder eine Aminosäuresequenz mit mindestens 70 % Sequenzidentität dazu umfasst, wobei ein Mangel an der Produktion des Polypeptids, das Steviolglycosidtransport vermittelt, durch Modifizieren, teilweises Deletieren oder vollständiges Deletieren einer für das Polypeptid kodierenden Nukleotidsequenz erzeugt wird, sodass im Wesentlichen keine Expression des Polypeptids in der Wirtszelle stattfindet.

4. Rekombinanter Wirt nach einem der vorhergehenden Ansprüche, umfassend eine oder mehrere rekombinante Nukleotidsequenzen, die kodieren für:

   ein Polypeptid mit ent-Copalylpyrophosphatsynthase-Aktivität;
   ein Polypeptid mit ent-Kaurensynthase-Aktivität;
   ein Polypeptid mit ent-Kaurenoxidase-Aktivität; und
   ein Polypeptid mit Kaurensäure-13-Hydroxylase-Aktivität.

5. Rekombinanter Wirt nach einem der vorhergehenden Ansprüche, umfassend eine rekombinante Nukleinsäuresequenz, die für ein Polypeptid mit NADPH-Cytochrom-P450-Reduktase-Aktivität kodiert.

6. Rekombinanter Wirt nach einem der vorhergehenden Ansprüche, umfassend eine rekombinante Nukleinsäuresequenz, die für eines oder mehrere der Folgenden kodiert:

   (i) ein Polypeptid mit UGT74G1-Aktivität;
   (ii) ein Polypeptid mit UGT2-Aktivität;
   (iii) ein Polypeptid mit UGT85C2-Aktivität; und
   (iv) ein Polypeptid mit UGT76G1-Aktivität.

7. Rekombinanter Wirt nach einem der Ansprüche 3 bis 6, wobei der rekombinante Wirt eine *Yarrowia lipolytica* ist.

8. Rekombinanter Wirt nach einem der vorhergehenden Ansprüche, wobei die Fähigkeit des Wirts, Geranylgeranyldiphosphat (GGPP) zu erzeugen hochreguliert wird.

9. Rekombinanter Wirt nach einem der vorhergehenden Ansprüche, umfassend eine Nukleinsäuresequenz, die für eines oder mehrere der Folgenden kodiert:

   ein Polypeptid mit Hydroxymethylglutaryl-CoA-Reduktaseaktivität; oder
   ein Polypeptid mit Farnesylpyrophosphatsynthetase-Aktivität.

10. Verfahren zum Erzeugen eines Steviolglycosids, umfassend das Fermentieren eines rekombinanten Wirts nach einem der vorhergehenden Ansprüche in einem geeigneten Fermentationsmedium und optional das Gewinnen des Steviolglycosids.

11. Verfahren nach Anspruch 10 zum Erzeugen eines Steviolglycosids, wobei das Verfahren im industriellen Maßstab durchgeführt wird.

Revendications

1. Hôte recombinant capable de produire un glycoside de stéviol qui surexprime un acide nucléique qui code pour un polypeptide qui effectue la médiation du transport de glycoside de stéviol et lequel polypeptide comprend la séquence d'acides aminés indiquée dans la SEQ ID NO: 29, ou une séquence d'acides aminés ayant une identité de séquence d'au moins 70% avec celle-ci, et dans lequel la quantité du glycoside de stéviol total à l'extérieur de la cellule, en comparaison avec l'intérieur de la cellule, est supérieure en comparaison avec une cellule correspondante qui ne surexprime pas un acide nucléique qui code pour un polypeptide qui effectue la médiation du transport de glycoside de stéviol et lequel polypeptide comprend la séquence d'acides aminés indiquée dans la SEQ ID NO: 29, ou une séquence d'acides aminés ayant une identité de séquence d'au moins 70% avec celle-ci, l'hôte recombinant étant une levure.

2. Hôte recombinant selon la revendication 1, l'hôte appartenant à l'un des genres *Saccharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Issatchenkia, Brettanomyces, Pachysolen* et *Yarrowia,* de préférence l'hôte recombinant étant une cellule de *Saccharomyces cerevisiae,* une cellule de *Yarrowia lipolytica,* une cellule de *Candida krusei* ou une cellule *d'Issatchenkia orientalis.*

3. Hôte Yarrowia recombinant capable de produire un glycoside de stéviol, qui a été modifié de préférence dans son génome, pour conduire à une déficience dans la production d'un polypeptide qui effectue la médiation du transport de glycoside de stéviol et lequel polypeptide comprend la séquence d'acides aminés indiquée dans la SEQ ID NO: 29, ou une séquence d'acides aminés ayant une identité de séquence d'au moins 70% avec celle-ci, et dans lequel la quantité du glycoside de stéviol total à l'extérieur de la cellule, en comparaison avec l'intérieur de la cellule, est inférieure en comparaison avec une cellule correspondante qui n'a pas une déficience dans la production d'un polypeptide qui effectue la médiation du transport de glycoside de stéviol et lequel polypeptide comprend la séquence d'acides aminés indiquée dans la SEQ ID NO: 29, ou une séquence d'acides aminés ayant une identité de séquence d'au moins 70% avec celle-ci, dans lequel une déficience dans la production dudit polypeptide effectuant la médiation du transport de glycoside de stéviol est générée en modifiant, en supprimant partiellement ou en supprimant totalement une séquence nucléotidique codant pour le polypeptide, de sorte que substantiellement aucune expression du polypeptide n'ait lieu dans la cellule hôte.

4. Hôte recombinant, selon l'une quelconque des revendications précédentes, qui comprend une ou plusieurs séquence(s) nucléotidique(s) recombinante(s) codant pour :

un polypeptide ayant une activité d'ent-copalyl pyrophosphate synthase ;
un polypeptide ayant une activité d'ent-Kaurène synthase ;
un polypeptide ayant une activité d'ent-Kaurène oxydase ; et
un polypeptide ayant une activité d'acide kauréno ïque 13 hydroxylase synthase.

5. Hôte recombinant, selon l'une quelconque des revendications précédentes, qui comprend une séquence recombinante d'acides nucléiques codant pour un polypeptide ayant une activité de NADPH-cytochrome p450 réductase.

6. Hôte recombinant, selon l'une quelconque des revendications précédentes, qui comprend une séquence recombinante d'acides nucléiques codant pour un ou plusieurs élément(s) parmi :

(i) un polypeptide ayant une activité d'UGT74G1 ;
(ii) un polypeptide ayant une activité d'UGT2 ;
(iii) un polypeptide ayant une activité d'UGT85C2 ; et
(iv) un polypeptide ayant une activité d'UGT76G1.

7. Hôte recombinant selon l'une quelconque des revendications 3 à 6, l'hôte recombinant étant une *Yarrowia lipolytica.*

8. Hôte recombinant selon l'une quelconque des revendications précédentes, dans lequel la capacité de l'hôte à produire du géranylgéranyl diphosphate (GGPP) est régulée à la hausse.

9. Hôte recombinant, selon l'une quelconque des revendications précédentes, qui comprend une séquence recombinante d'acides nucléiques codant pour un ou plusieurs élément(s) parmi :

un polypeptide ayant une activité d'hydroxyméthylglutaryl-CoA réductase ; ou
un polypeptide ayant une activité de farnésyl-pyrophosphate synthétase.

10. Processus destiné à la préparation d'un glycoside de stéviol qui comprend une fermentation d'un hôte recombinant, selon l'une quelconque des revendications précédentes, dans un milieu de fermentation approprié et, en option, une récupération du glycoside de stéviol.

11. Processus, selon la revendication 10, destiné à la préparation d'un glycoside de stéviol, le processus étant effectué à une échelle industrielle.

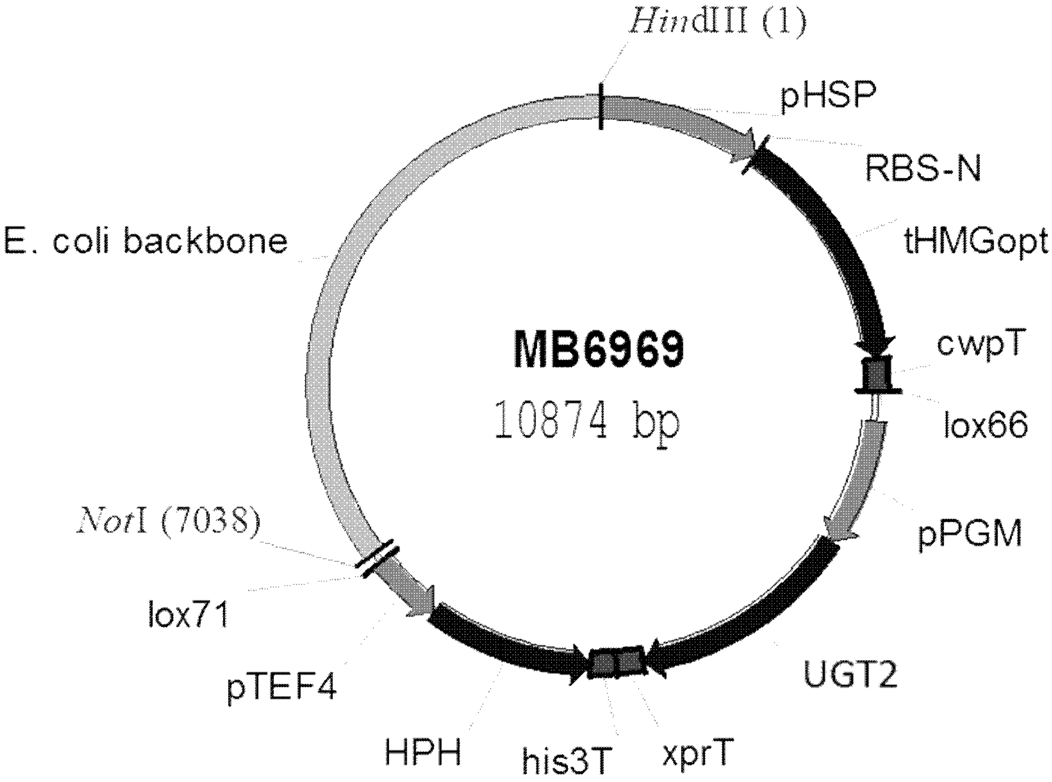

*Hin*dIII (1)
pHSP
RBS-N
tHMGopt
cwpT
lox66
pPGM
UGT2
xprT
his3T
HPH
pTEF4
lox71
*Not*I (7038)
E. coli backbone
MB6969
10874 bp

Fig. 1

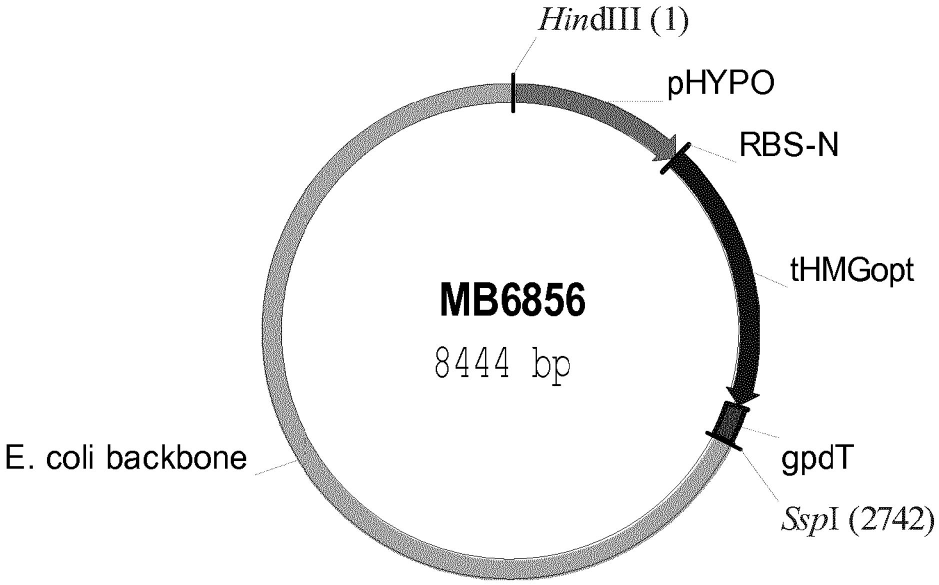

*Hin*dIII (1)
pHYPO
RBS-N
tHMGopt
MB6856
8444 bp
gpdT
*Ssp*I (2742)
E. coli backbone

Fig. 2

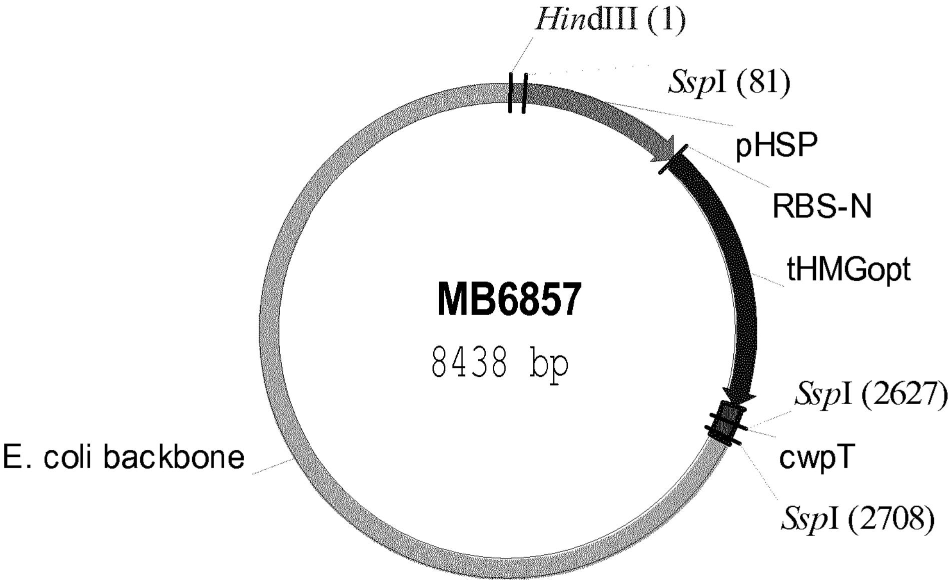

*Hin*dIII (1)
*Ssp*I (81)
pHSP
RBS-N
tHMGopt
MB6857
8438 bp
*Ssp*I (2627)
cwpT
*Ssp*I (2708)
E. coli backbone

Fig. 3

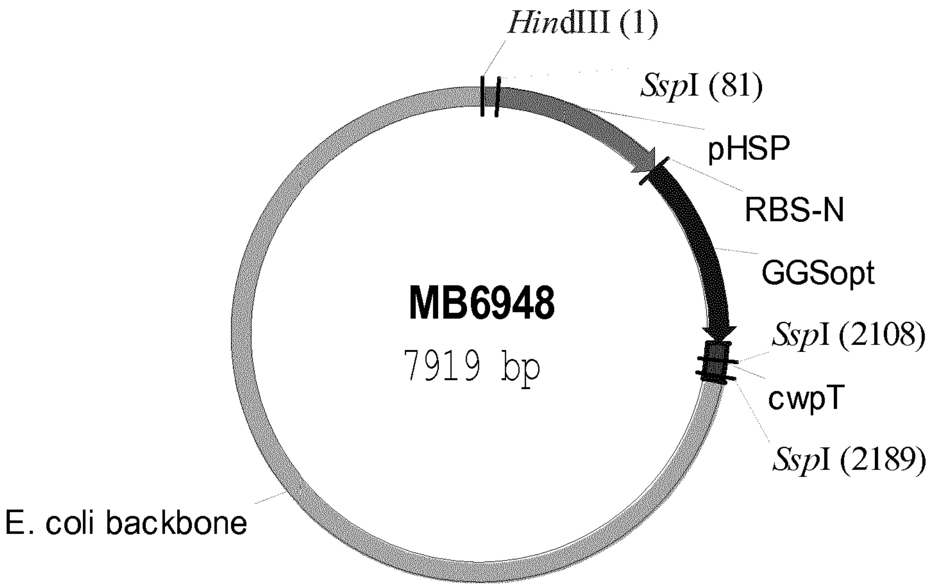

*Hin*dIII (1)
*Ssp*I (81)
pHSP
RBS-N
GGSopt
*Ssp*I (2108)
cwpT
*Ssp*I (2189)
E. coli backbone
MB6948
7919 bp

Fig. 4

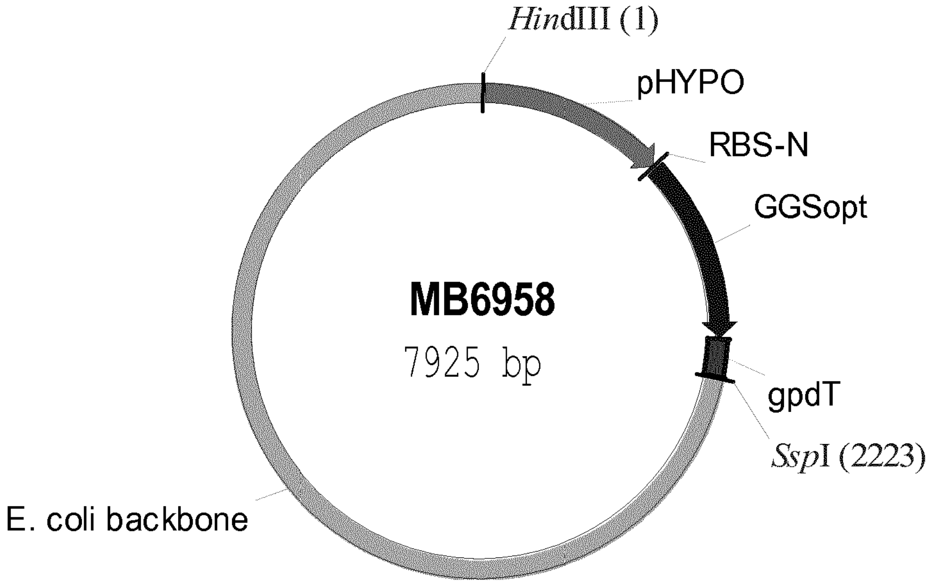

*Hin*dIII (1)
pHYPO
RBS-N
GGSopt
MB6958
7925 bp
gpdT
*Ssp*I (2223)
E. coli backbone

Fig. 5

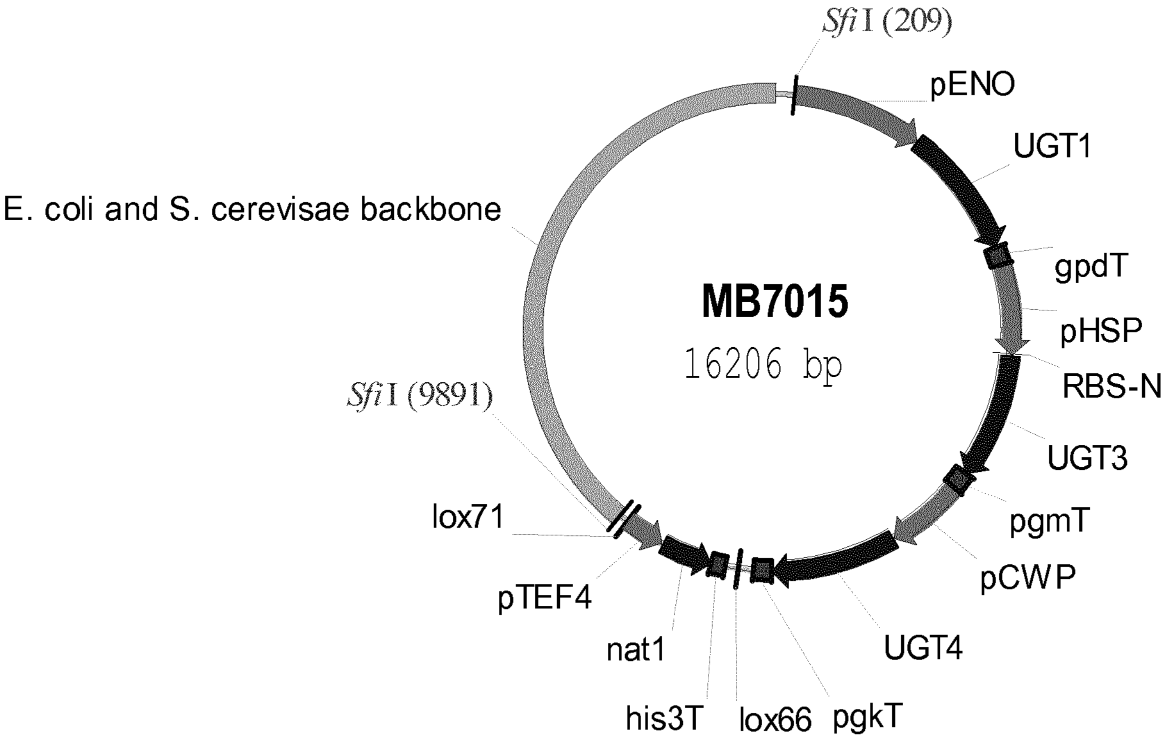

SfiI (209)
pENO
UGT1
E. coli and S. cerevisae backbone
gpdT
MB7015
16206 bp
pHSP
RBS-N
SfiI (9891)
UGT3
pgmT
lox71
pCWP
pTEF4
UGT4
nat1
his3T
lox66
pgkT

Fig. 6

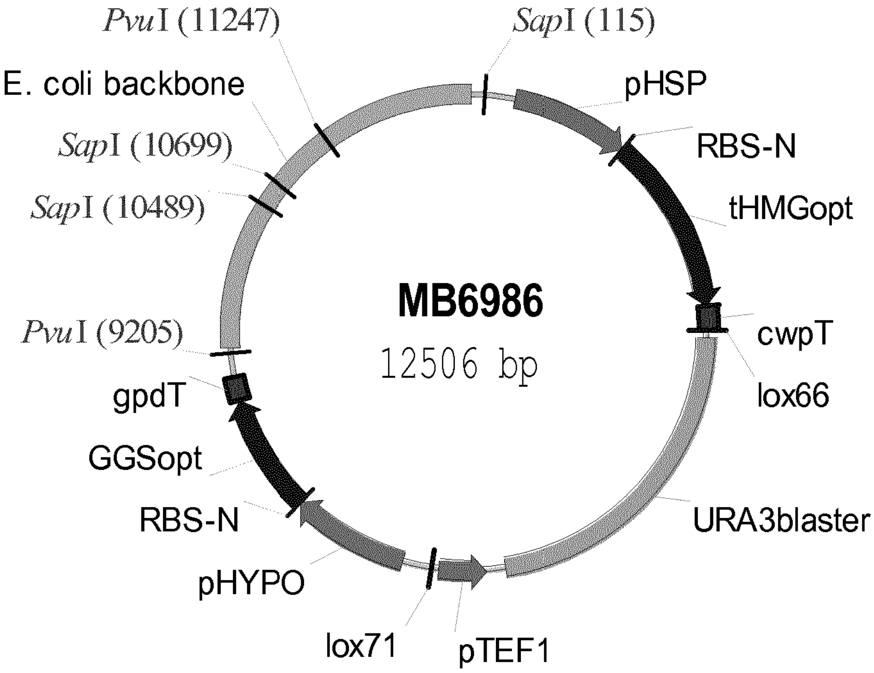

*Pvu*I (11247)
*Sap*I (115)
E. coli backbone
pHSP
*Sap*I (10699)
RBS-N
*Sap*I (10489)
tHMGopt
MB6986
12506 bp
*Pvu*I (9205)
cwpT
gpdT
lox66
GGSopt
RBS-N
URA3blaster
pHYPO
lox71
pTEF1

Fig. 7

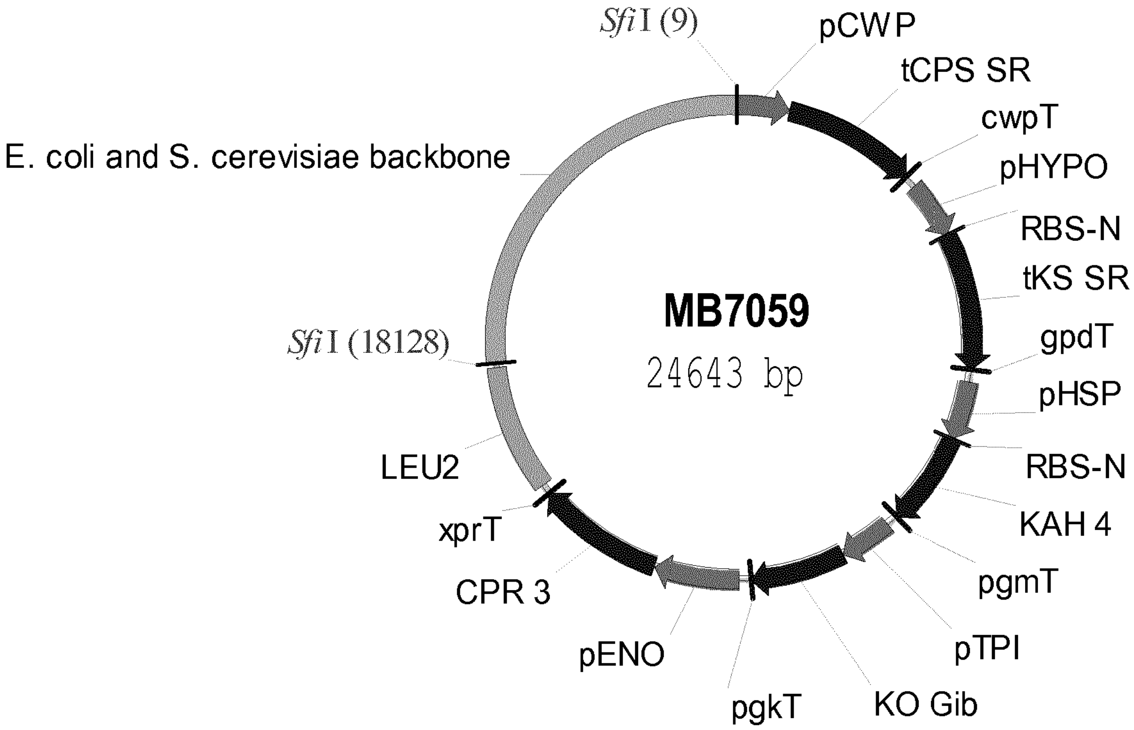

*Sfi*I (9)
pCWP
tCPS SR
cwpT
pHYPO
RBS-N
tKS SR
gpdT
pHSP
RBS-N
KAH 4
pgmT
pTPI
KO Gib
pgkT
pENO
CPR 3
xprT
LEU2
*Sfi*I (18128)
E. coli and S. cerevisiae backbone
MB7059
24643 bp

Fig. 8

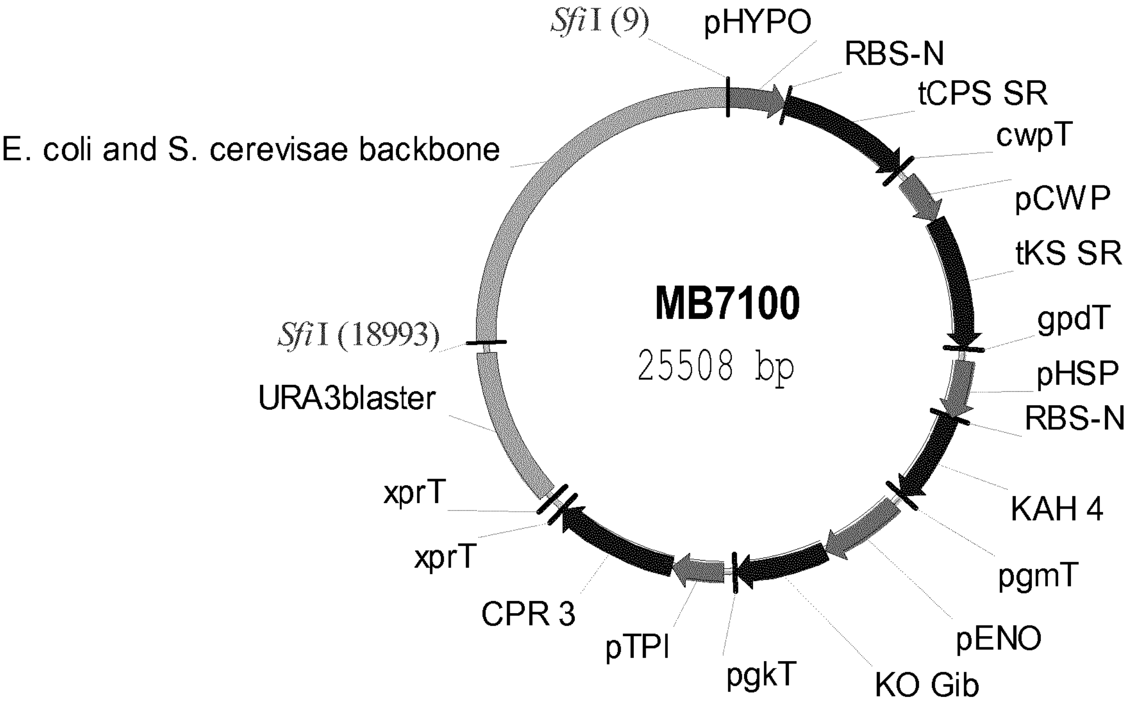

*Sfi*I (9)
pHYPO
RBS-N
tCPS SR
cwpT
pCWP
tKS SR
gpdT
pHSP
RBS-N
KAH 4
pgmT
pENO
KO Gib
pgkT
pTPI
CPR 3
xprT
xprT
URA3blaster
*Sfi*I (18993)
E. coli and S. cerevisae backbone
MB7100
25508 bp

Fig. 9

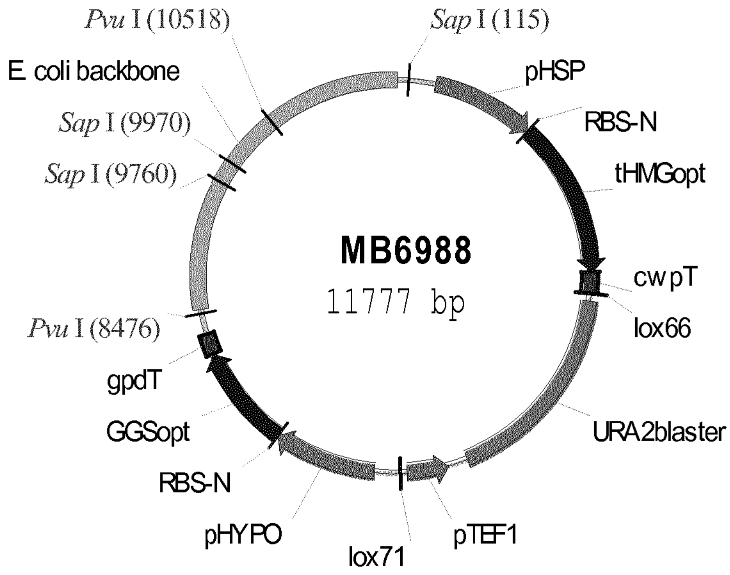

Pvu I (10518)
Sap I (115)
E. coli backbone
pHSP
Sap I (9970)
RBS-N
Sap I (9760)
tHMGopt
MB6988
11777 bp
cwpT
Pvu I (8476)
lox66
gpdT
GGSopt
URA2blaster
RBS-N
pHYPO
lox71
pTEF1

Fig. 10

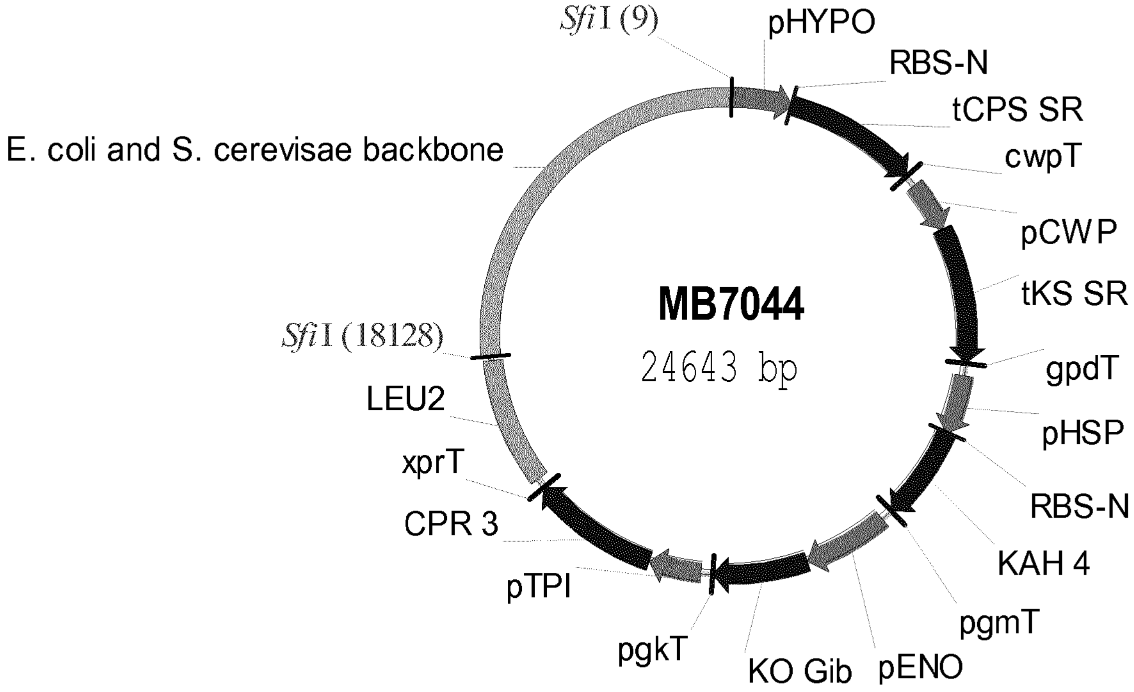

*Sfi*I (9)
pHYPO
RBS-N
tCPS SR
cwpT
pCWP
tKS SR
gpdT
pHSP
RBS-N
KAH 4
pgmT
pENO
KO Gib
pgkT
pTPI
CPR 3
xprT
LEU2
*Sfi*I (18128)
E. coli and S. cerevisae backbone
MB7044
24643 bp

Fig .11

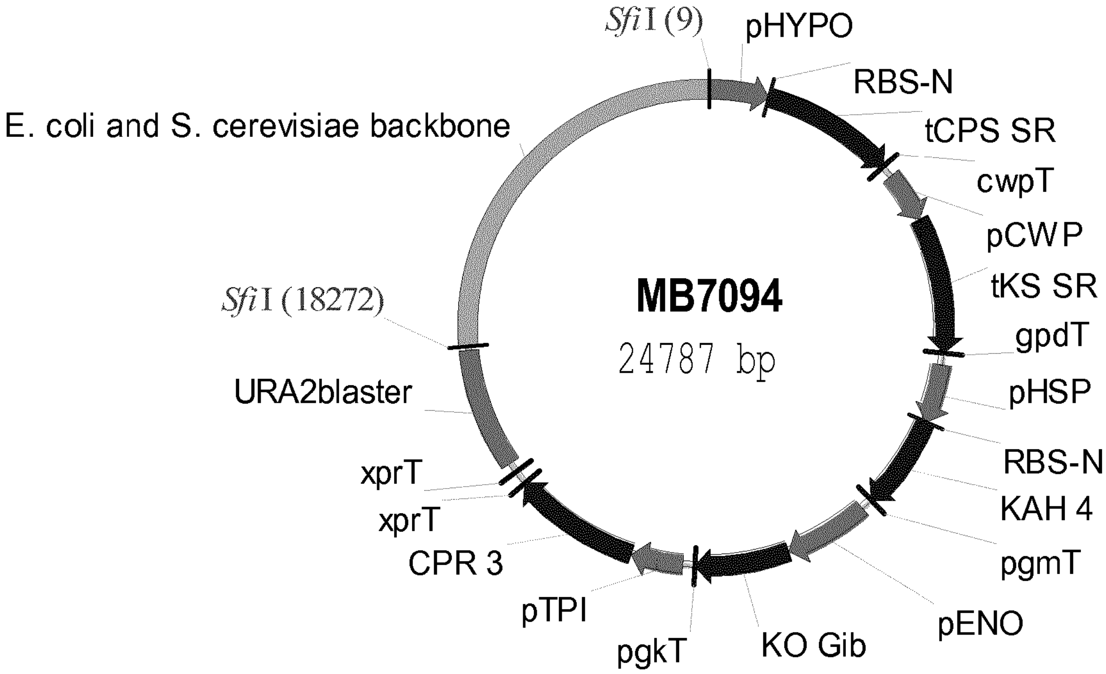

SfiI (9)
pHYPO
RBS-N
tCPS SR
cwpT
pCWP
tKS SR
gpdT
pHSP
RBS-N
KAH 4
pgmT
pENO
KO Gib
pgkT
pTPI
CPR 3
xprT
xprT
URA2blaster
SfiI (18272)
E. coli and S. cerevisiae backbone
MB7094
24787 bp

Fig. 12

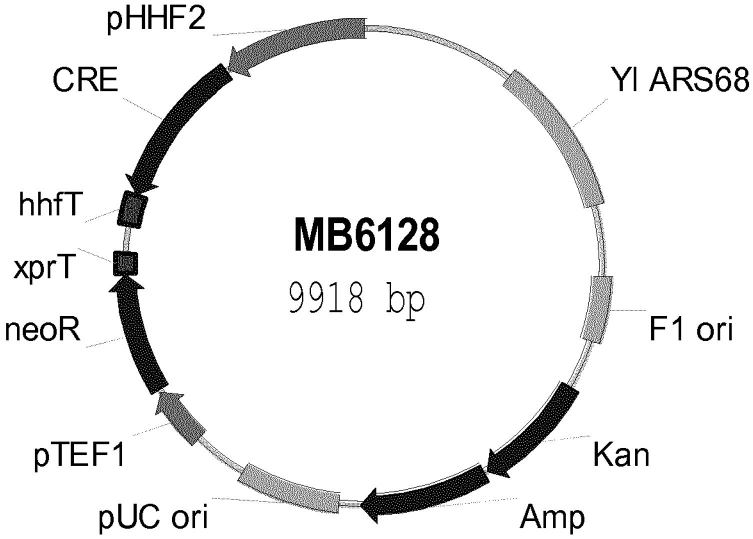
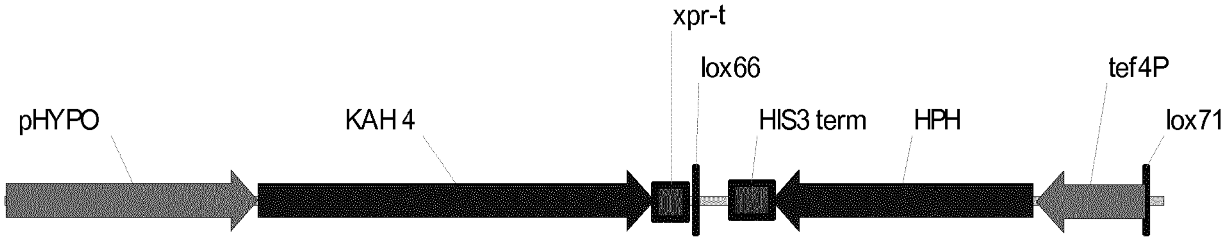

pHHF2
CRE
hhfT
xprT
neoR
pTEF1
pUC ori
Amp
Kan
F1 ori
Yl ARS68
MB6128
9918 bp

Fig. 13



pHYPO-KAH/HYG-R PCR product
4614 bp

Fig. 14

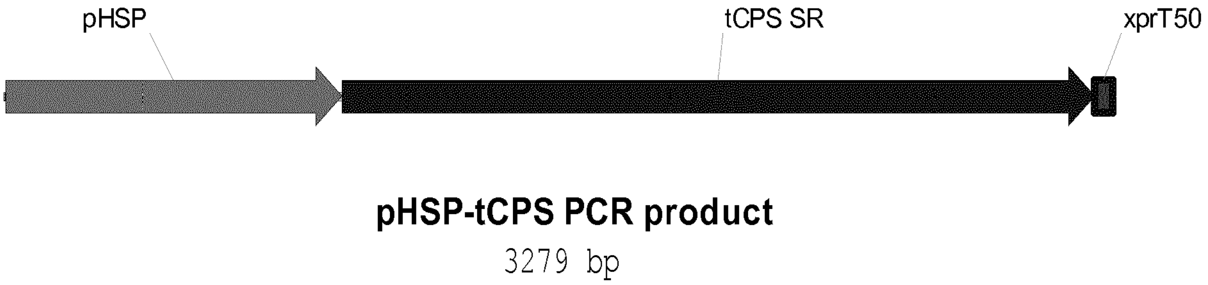


Fig. 15

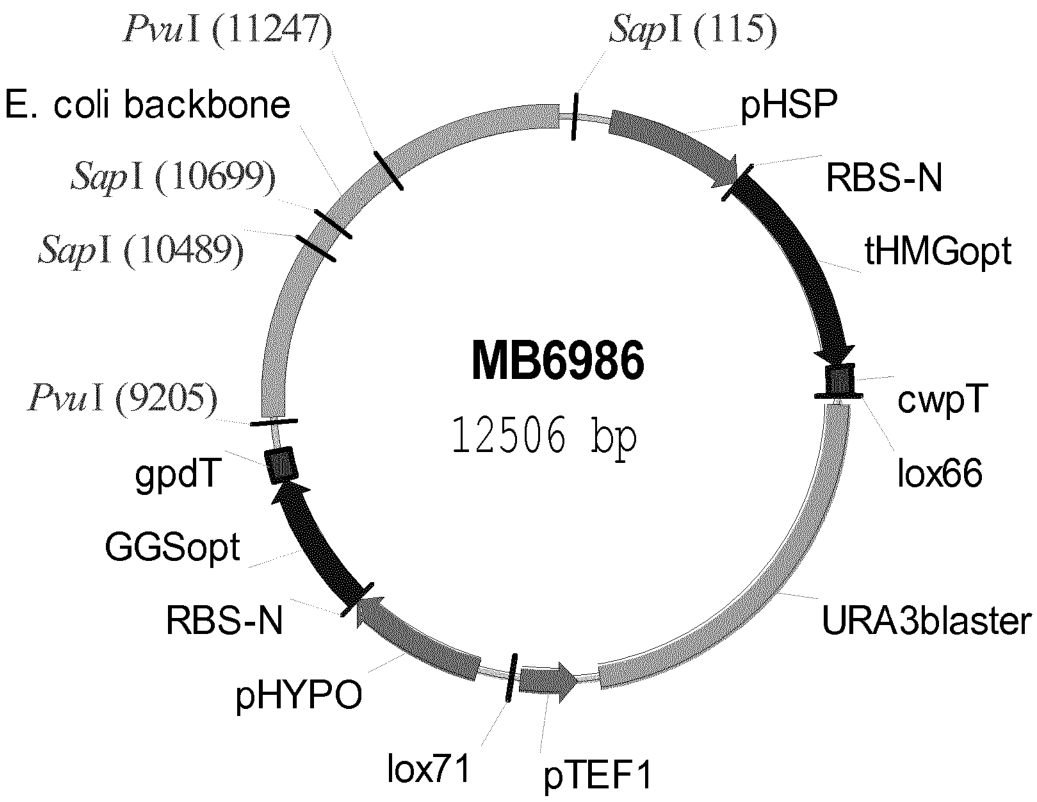

PvuI (11247)
SapI (115)
E. coli backbone
pHSP
SapI (10699)
RBS-N
SapI (10489)
tHMGopt
MB6986
12506 bp
PvuI (9205)
cwpT
gpdT
lox66
GGSopt
RBS-N
URA3blaster
pHYPO
lox71
pTEF1

Fig. 16

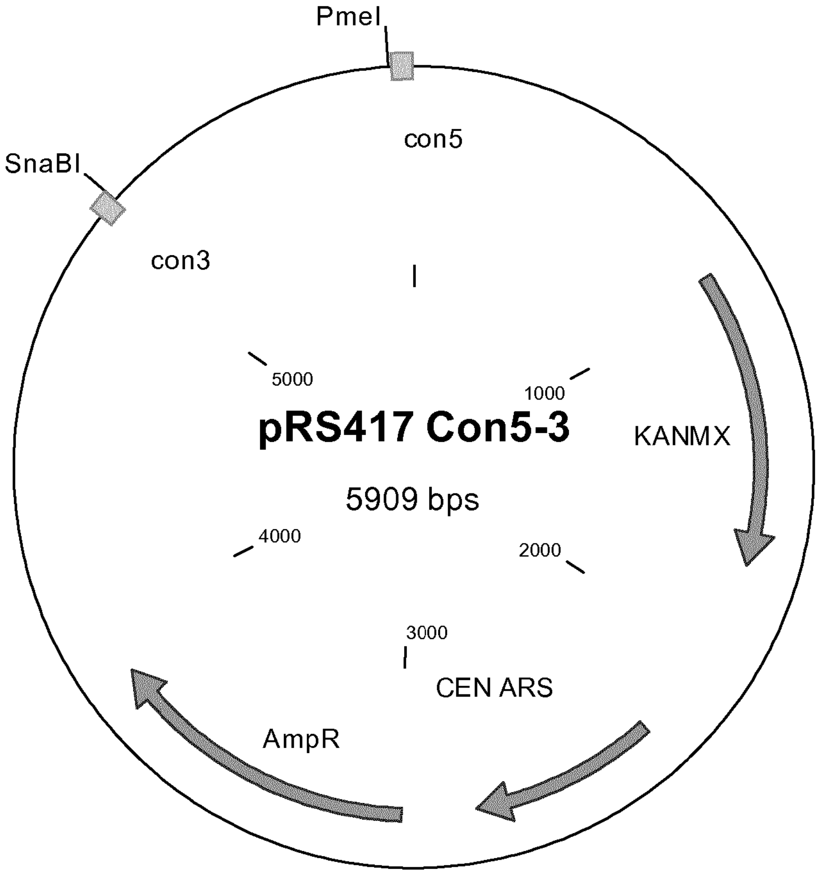

PmeI
SnaBI
con5
con3
5000
1000
pRS417 Con5-3
5909 bps
KANMX
4000
2000
3000
CEN ARS
AmpR

Fig. 17

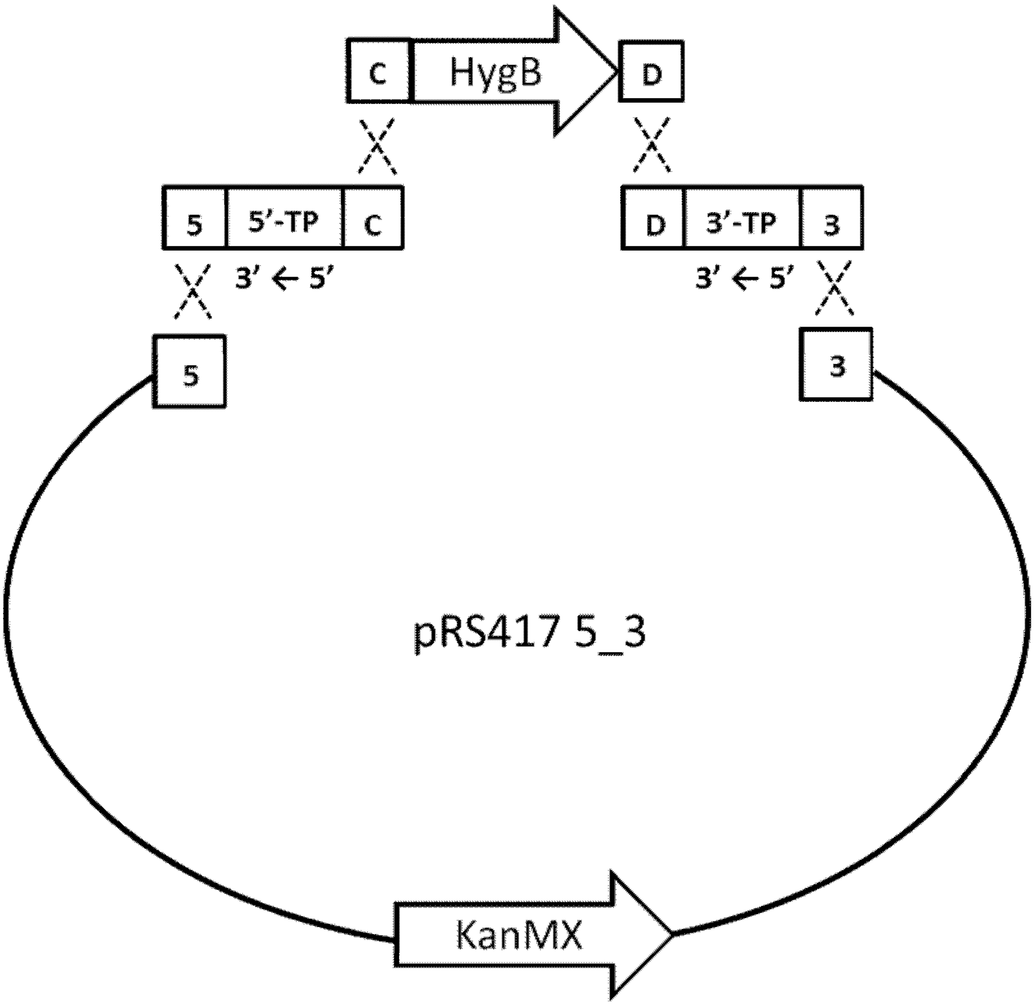


Fig. 18

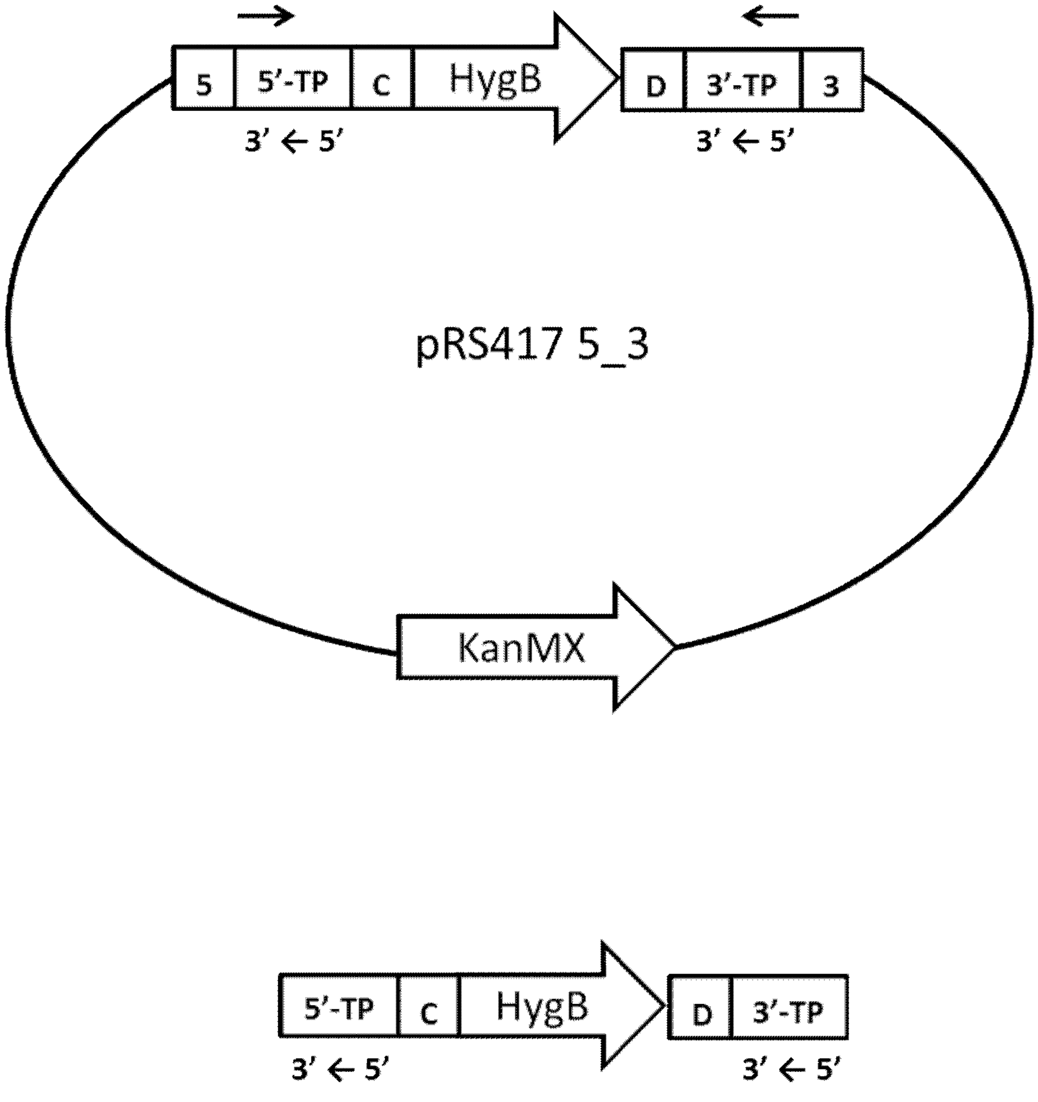


Fig. 19

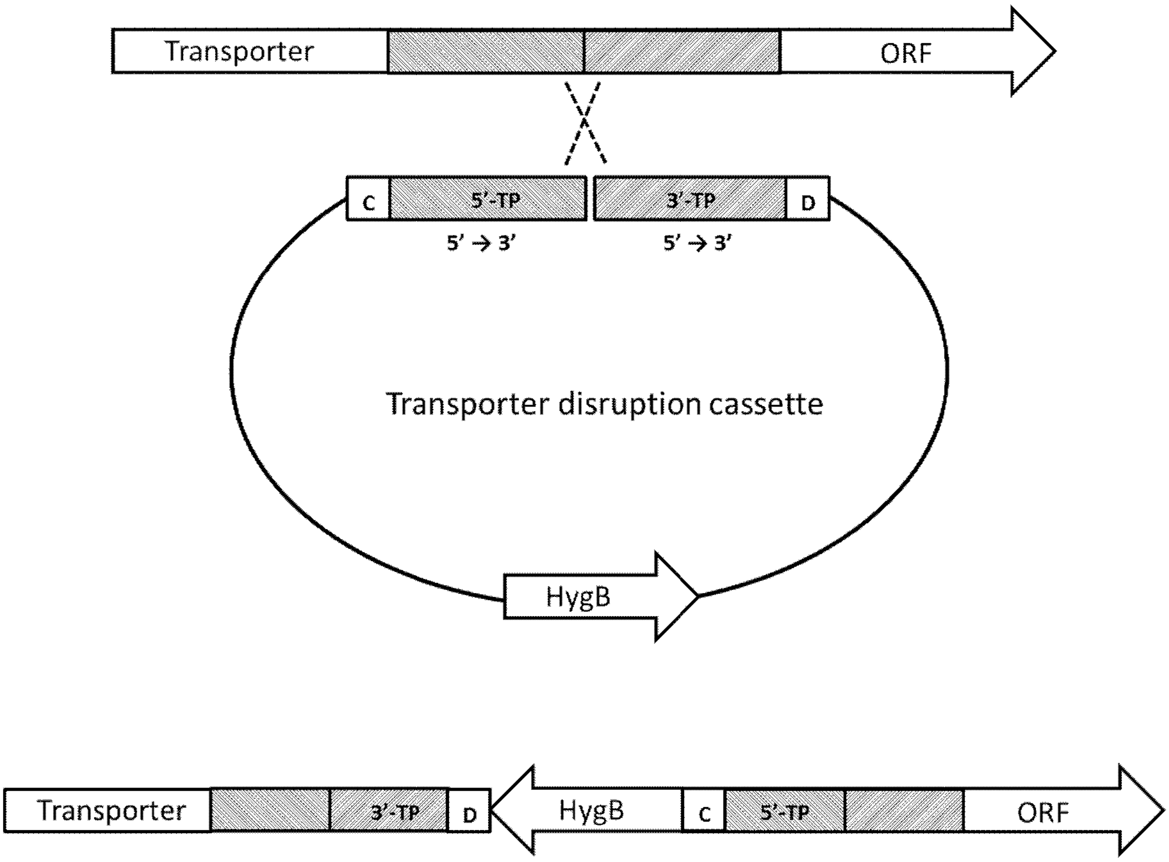

Transporter
ORF
C
5'-TP
3'-TP
D
5' → 3'
5' → 3'
Transporter disruption cassette
HygB
Transporter
3'-TP
D
HygB
C
5'-TP
ORF

Fig. 20

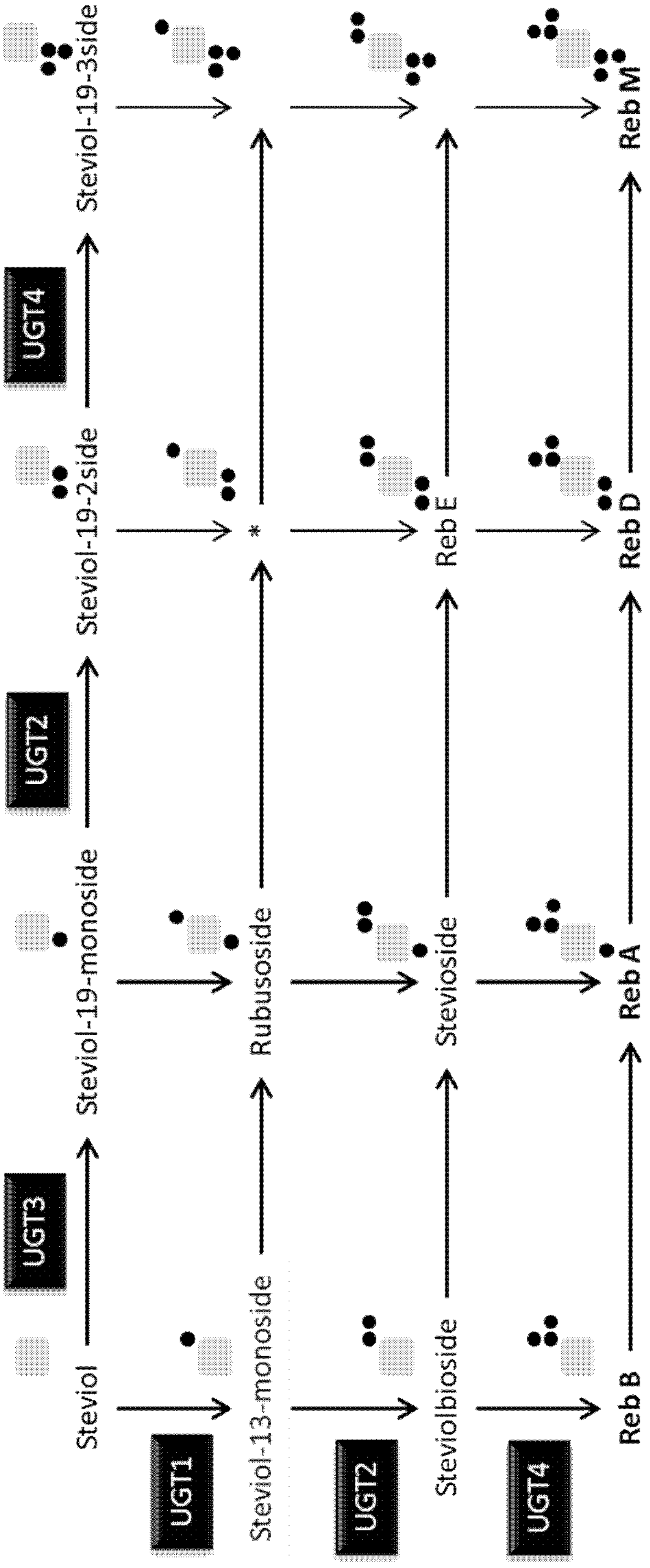

Steviol
UGT3
Steviol-19-monoside
UGT2
Steviol-19-2side
UGT4
Steviol-19-3side
UGT1
Steviol-13-monoside
Rubusoside
*
UGT2
Steviolbioside
Stevioside
Reb E
UGT4
Reb B
Reb A
Reb D
Reb M

Fig. 21

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- WO 06009434 A **[0127]**
- EP 1499708 B1 **[0127]**
- WO 2006096130 A **[0127]**
- WO 04099381 A **[0127]**
- EP 0635574 A **[0170] [0173]**
- WO 9846772 A **[0170] [0173]**
- WO 9960102 A **[0170] [0173]**
- WO 0037671 A **[0170] [0173]**
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- **SAMBROOK ; RUSSEL.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 2001 **[0170] [0173]**
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